The Enzymatic and Chemical Synthesis of Nucleoside Analogues using N-Deoxyribosyltransferase from *Lactobacillus leichmannii*

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To Mum,

"The impossible often has a kind of integrity to it which the merely improbable lacks. How often have you been presented with an apparently rational explanation of something which works in all respects other than one, which is just that it is hopelessly improbable?"

The Long Dark Tea-Time Of The Soul Douglas Adams

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DECLARATION

The work described in this thesis is the original of the author, except where acknowledgement has been made to results and ideas previously published. The work was carried out at the Department of Chemistry, University of Warwick, between October 1st 1989 and September 30th 1992 and has not previously been submitted for a degree at any institution.

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ABSTRACT

N–deoxyribosyltransferase catalyses the transfer of 2-deoxy-, 2,3-dideoxy- and 2,5-dideoxyribose sugars between purine and pyrimidine bases. In this work, a crude extract of N-deoxyribosyltransferase from *Lactobacillus leichmannii* was used in the chemoenzymatic synthesis of nucleoside analogues with potential antiviral activity, in particular, 9-β-D-2′,3′-dideoxyribofuranosyl 2-aminopurine which had anti–HIV activity.

The immobilisation of a crude extract of N-deoxyribosyltransferase on a variety of support matrices is described. The most successful matrices were octyl Sepharose and Poly(acrylamide-co-N-acryloxysuccinimide) (PAN-550) gel. However, the PAN method gave higher and more sustained levels of activity, was easier to handle and incubate, and provided a general batch reactor method for the large scale synthesis of nucleosides such as 2-thio-2′-deoxyuridine.

As the synthesis of 3′-substituted-2′,3′-dideoxynucleosides was not possible by the enzymatic transfer method, an investigation into the conformations adopted by the nucleosides, which were and were not glycosyl donor nucleosides in the transfer reaction, was undertaken using variable temperature ^{1}H NMR. Little is known about the active sites of the N-deoxyribosyltransferases from lactobacilli, but major factors accounting for this lack of reactivity may be steric hindrance or dipolar effects that inhibit the binding of a substrate to the transferase. Another factor which may be important is the conformation and flexibility of the deoxyribose ring, because a substrate which adopts a "rigid", unfavourable shape may not bind to the active site of the transferase and may be inactive as a substrate. Analysis of the variable temperature ^{1}H NMR data led to information about the conformer populations, the equilibrium constants and, the free energy, enthalpy and entropy of each system. The temperature dependance of the coupling constants yielded quantitative information with regard to the position of the dynamic equilibrium between the two principal conformers of the sugar ring and determined the flexibility of sugar rings in nucleosides. It was found that only "flexible" sugar rings without extremes of conformation were active as glycosyl donors in our system.

The modified nucleoside, (±)-1-[(2′β,4′β)-2′-(hydroxymethyl)-4′-dioxolanyl]thymine, [(±)-Dioxolane–T], in which the 3′-carbon atom is replaced by oxygen, was prepared. Its ability to act as a glycosyl donor for N-deoxyribosyltransferases from lactobacillus was investigated but unfortunately its extreme conformation prevented it from being an active glycosyl donor.

ABBREVIATIONS

AIBN	Azobis(isobutyronitrile)
AIDS	Aquired Immune Deficiency Syndrome
ATCC	American Type Culture collection
AZT	3'-Azido-3'-deoxythymidine
b.p.	Boiling point
CI	Chemical ionisation
CMV	Cytomegalovirus
DAST	Diethylaminosulphur trifluoride
DMAP	4-(Dimethylamino)pyridine
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EBV	Epstein Barr Virus
EI	Electron impact
g	grams
h	hours
HIV	Human Immunodeficiency Virus
HPLC	High pressure liquid chromatography
HSV	Herpes Simplex Virus
Hz	Hertz
J	Coupling constant
K_{eq}	Equilibrium constant
MS	Mass spectrometry
m.p.	Melting point
mg	Milligrams
min	minutes
ml	Millilitre
nm	Nanometres

NMR	Nuclear magnetic resonance spectroscopy
nOe	Nuclear Overhauser enhancement
PAN	Poly(acrylamide-co-N-acryloxysuccinimide)
PDC	Pyridinium dichromate
PIPES	[1,4-piperazinebis(ethanesulphonic acid)]
ppm	Parts per million
RNA	Ribonucleic Acid
rpm	Revolutions per minute
sec	seconds
TET	Triethylenetetramine
THF	Tetrahydrofuran
tlc	Thin-layer chromatography
TMS	Tetramethylsilane
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultra-violet
VZV	Varicella Zoster Virus
°C	Degrees Centigrade

Ac	Acetyl
R	Alkyl
Me	Methyl
Et	Ethyl
Bn	Benzyl
Bz	Benzoyl
MMTr	Monomethoxytrityl
Tr	Trityl

CHAPTER 1

INTRODUCTION

1.1: Background

Viruses were discovered in the 1890's following studies on the tobacco leaf mosaic disease[1]. At that time bacteria were believed to be the organisms responsible for disease. However, it was found bacteria-free filtrates were still able to induce disease in healthy plants and the causative agents were called viruses.

In 1898 "Filterable Viruses" were confirmed when foot and mouth disease was transmitted from the bacteria-free lymph of infected cattle[2]. Viral infections such as influenza, herpes, hepatitis, rabies, chicken pox, and mumps account for about sixty per cent of all illness in developed countries. In 1941 the introduction of penicillin transformed medicine, giving physicians a powerful and versatile weapon against infectious bacteria, which only account for fifteen per cent of all diseases. Over forty years on there is still no comparable remedy for viral infections, but the threat of other viruses has been overshadowed by the spread of aquired immune deficiency syndrome (AIDS).

Viruses consist of a genetic component (DNA or RNA) contained in a protein coat (capsid) made up of glycoproteins and/or lipids. The structure of the viral coat, type of nuclei acid, mode of entry into cells and the mechanism of replication all vary depending on the virus.

There are few effective treatments for viral diseases (none for retroviruses) since viruses (unlike bacteria, which are independent of the host cell with unique enzymes and substrates and therefore effective therapies can be targetted) always commandeer the host cells' genetic machinery in order to reproduce. This obligate, intracellular nature of viruses produces an intimate involvement between host and virus which makes selective inhibition of viral replication difficult without affecting the host cells. Indeed, until the beginning of the 1960's the problem of distinguishing viral functions from cellular ones for selective attack of the virus was thought to be insurmountable. Instead the main strategy for controlling viral infections was (and still is to a large extent) the development of vaccines, which forestall infection by stimulating the immune system in advance rather than attack the virus directly.

1.2: Antiviral Therapy

Since the advent of AIDS, first described in the early 1980's,[3] the search for new antiviral drugs has intensified rapidly. As yet there is no cure for AIDS or a vaccine for the Human Immunodeficiency Virus (HIV) which is the cause of the disease and the epidemic continues to spread. Therefore, there is an increased demand for more potent drugs, not only to inhibit and prevent the replication of the HIV, but also to treat the many life threatening opportunistic infections associated with this immunosuppressive condition[3, 4].

An effective antiviral agent must either kill or stop the pathogen from multiplying without harming the infected host significantly. Selective agents exert their favourable effects through one or more of the following:

- they are accumulated principally by the infected cell;
- they utilise differences in the systems: attacking a biochemical pathway that is unique to the pathogen or infected cell;
- they react exclusively with a structure that exists only in the virus.

The replication of viruses is intimately connected with the biochemical mechanisms of the host cell and so there are few unique functions for selective attack. In the replication of viral nucleic acids there exist certain enzymes that are specifically coded for by the virus. These processes are therefore unique to infected cells and so offer an ideal opportunity for selective chemotherapy[5, 6].

1.3: Nucleoside Analogues

1.3.1: Inhibitors of DNA Syntheses

Herpes viruses are a class of double-stranded DNA viruses which cause a variety of diseases in humans: e.g. cold sores, eye infections (keratitis), genital sores, chicken-pox, shingles and glandular fever (infectious mononucleosis). The different viruses include herpes simplex virus (HSV-1 and HSV-2), Varicella zoster virus (VZV), cytomegalovirus (CMV), and Epstein Barr virus (EBV)[7-10].

There are many nucleoside analogues targetted at the herpes virus because it codes for many enzymes required in its replication and metabolism[11, 12]. This produces some selectivity for the drug action. An example of an effective drug is 9-(2-hydroxyethoxymethyl)guanine (ACV, acyclovir, Fig. 1.1)[13, 14].

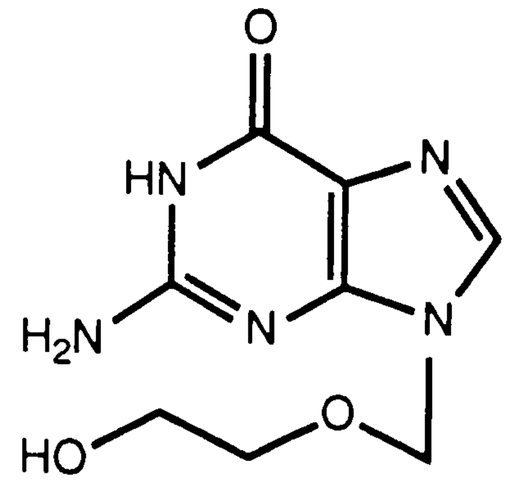


Fig. 1.1 The chemical structure of Acyclovir

Acyclovir has a low toxicity as it is only metabolised in cells infected with the herpes viruses, where it has a much higher affinity for the virus induced deoxythymidine kinase than for the corresponding kinase of the host cells. This kinase phosphorylates the acyclovir into acyclovir monophosphate which is further phosphorylated into the triphosphate. Once again the DNA polymerase of the herpes virus has a much higher affinity for the acyclovir triphosphate than the cellular polymerase; hence they are added to viral DNA in preference to cellular DNA. The drug is incorporated into the growing DNA strand where it acts as a chain terminator due to the lack of the 3′-hydroxyl group. Furthermore, it also acts as a suicide substrate for the DNA polymerase[15-17].

However, as the replication cycle of each class of virus is unique there are few broad-spectrum antiviral agents, unlike the broad spectrum antibiotics. An example is phosphonoformate[18]. Any drug which affects more than a single viral species is likely to impair a fundamental biochemical pathway and therefore have inevitable host cell toxicity.

There are only a few antiviral drugs licensed for use:

DRUG	VIRUS
Amantadine	Influenza A
Rimantadine	Influenza A (USSR)
5-Iodo-2′-deoxyuridine	HSV
5-Trifluoromethyl-2′-deoxyuridine	HSV
Adenine arabinoside	HSV
Acyclovir	HSV, VZV
Gancyclovir	Cytomegalovirus
5-Ethyl-2′-deoxyuridine	HSV (Germany)
5-Iodo-2′-deoxycytidine	HSV (France)
Ribavirin	Respiratory syncytial virus
3′-Azido-2′,3′-dideoxythymidine	HIV-1
2′,3′-dideoxyinosine	HIV-1

Table 1.1 Licensed antiviral drugs[19]

Most of the promising drugs have been nucleoside analogues and the majority of current successes have been centered around interrupting nucleic acid synthesis[11, 15, 20-23].

There are many other antiviral compounds which owe their effects to their resemblance to nucleosides. They can act as chain terminators[24-26], inhibit the viral-specific mRNA capping enzymes, guanyl transferase and N-7 methyl transferase[20, 21, 27], or they can be suicide substrates for some polymerases[13].

1.4: The AIDS Virus

Aquired immune deficiency syndrome (AIDS) is believed to have spread from central Africa to the Caribbean and then the U.S. and Europe. In 1984 the disease was shown to be caused by a retrovirus known as the human immunodeficiency virus (HIV)[3].

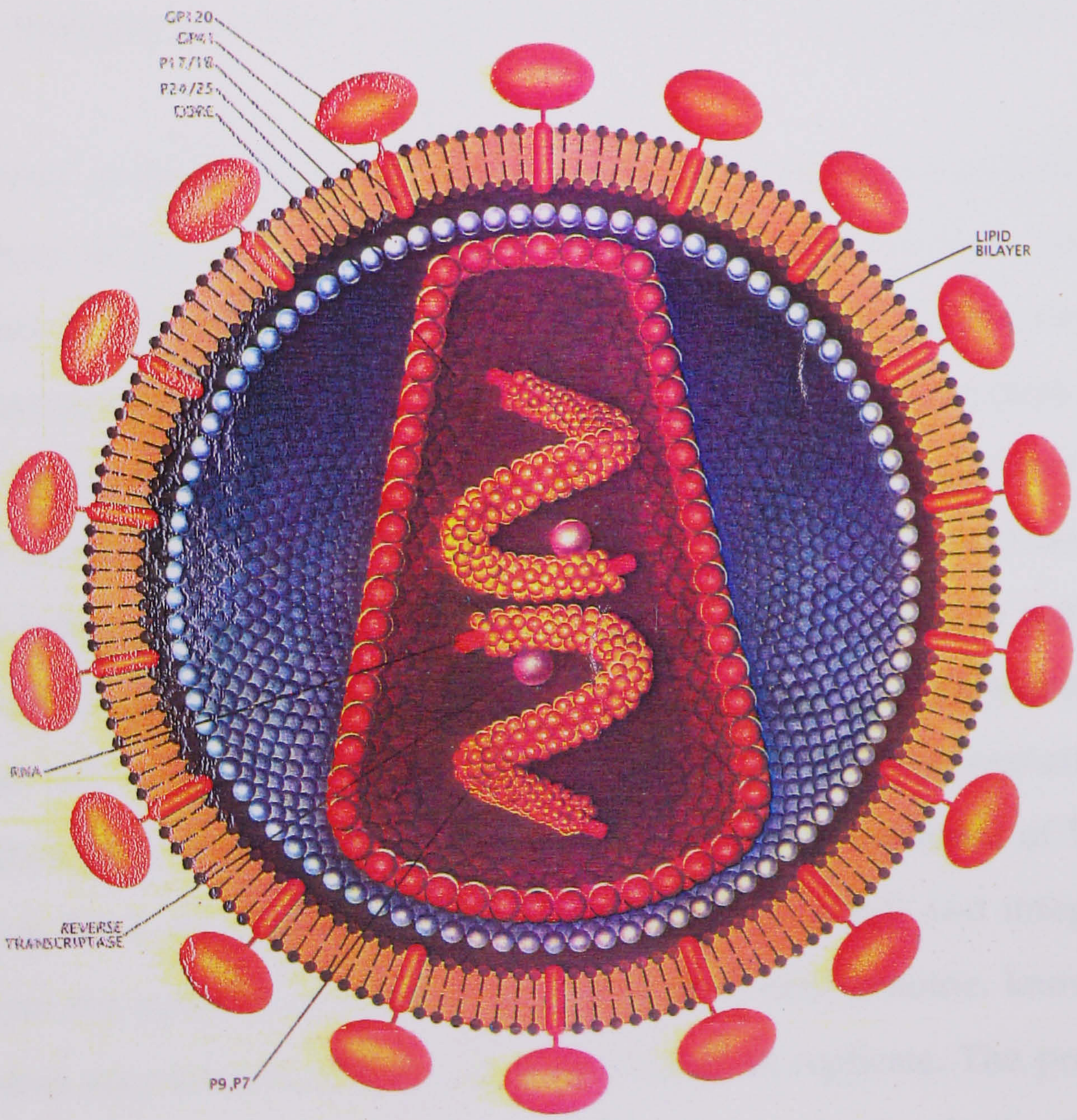


Fig. 1.2 Diagramatic representation of HIV[28]

HIV is a member of the lentivirus family (a sub-class of retrovirus) and like other viruses, cannot replicate without taking over the biosynthetic

apparatus of the host cell and exploiting it for its own ends. The HIV virion is a sphere that is roughly 1000 Å units across. The virus particle is covered by a membrane, made up of two layers of lipid material, that is derived from the outer membrane of the host cell. Studding the membrane are glycoproteins: each has two components; *gp*41 spans the membrane and *gp*120 extends beyond it. The membrane-and-protein envelope covers a core made up of proteins designated *p*24 and *p*18. The viral RNA is carried in the core, along with several copies of the enzyme reverse transcriptase, which catalyzes the assembly of the viral DNA[28].

The virus possesses no energy-producing or protein-synthesizing machinery of its own. In order to reproduce, it must therefore become absorbed to a host cell on specific cell-surface receptor molecules, be transported across the cell membrane, penetrate the cell cytoplasm, and uncoat in order that its viral genes can be expressed. The genome may require transportation to the cytoplasm or the nucleus. In retroviruses the genetic information is encoded on RNA and in order for them to replicate this must be transcribed into DNA. Once the RNA has been injected into the host cell, a virally encoded enzyme, reverse transcriptase, exploits the viral RNA as a template to assemble a corresponding molecule of DNA. The viral DNA then travels to the nucleus of the host cell and integrates itself into the genome of the host. The integrated viral genome, known as a provirus may remain latent until it is activated to replicate. The proviral DNA is then transcribed into mRNA, which directs the synthesis of viral proteins. The provirus also gives rise to other RNA copies which will serve as the genetic material of viral progeny. The proteins and the genomic RNA congregate at the cell membrane and assemble to form new HIV particles, which then bud from the cell[16].

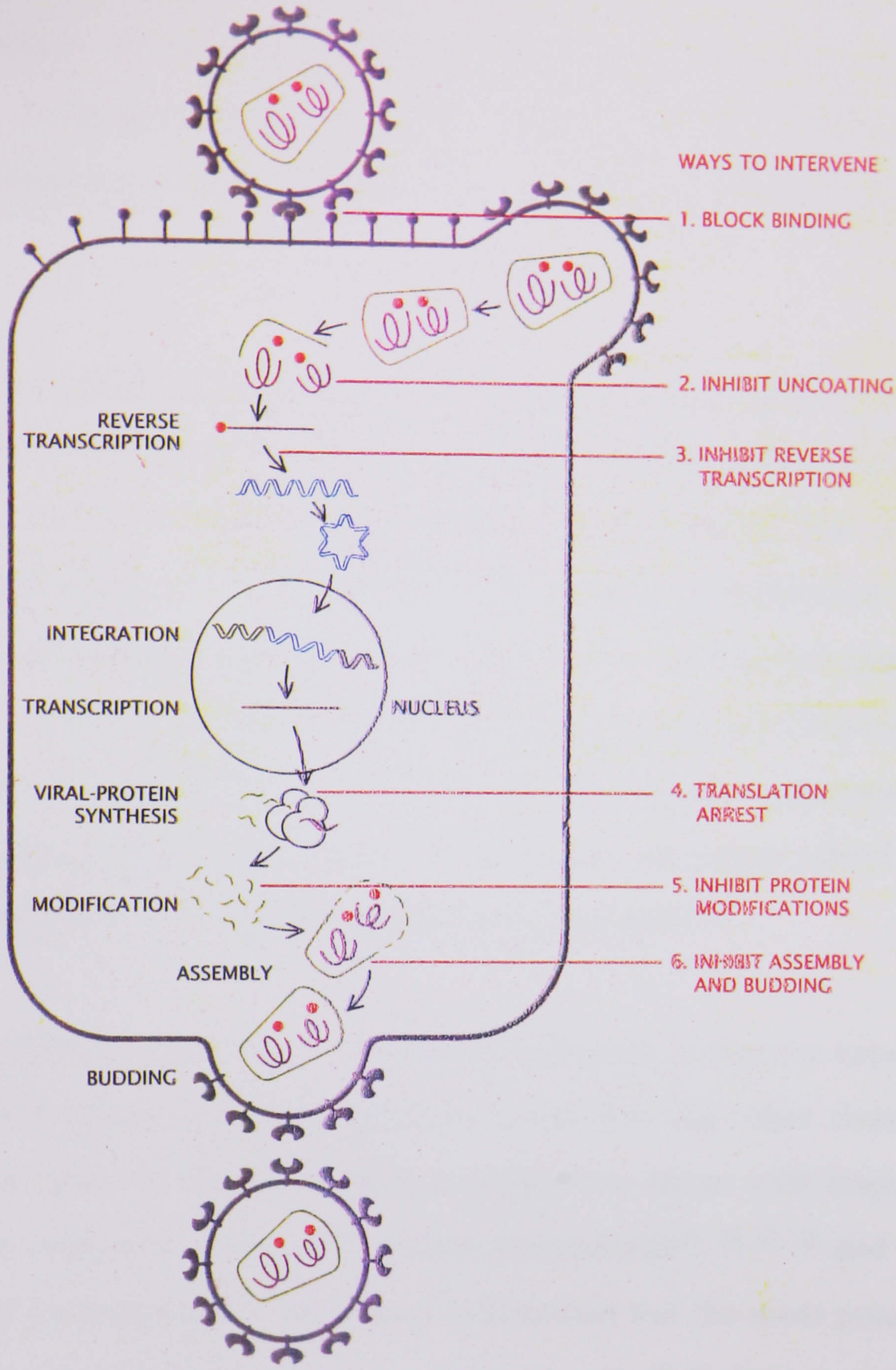


Fig. 1.3 Stages in the life-cycle of HIV which can be targetted by drugs[29]

1.5: AIDS Therapy

The wealth of knowledge that has now been gained about HIV enables the groundwork for treatment and prevention to be laid. The HIV life-cycle offers many potential targets for antiviral agents to inhibit such as:

adsorption, penetration, uncoating, reverse transcription, proviral DNA circularization and integration, viral mRNA transcription, maturation (capping, splicing, polyadenylation), and translation, viral protein precursor cleavage and glycosylation, virus assembly and release (budding)

30-34

The most tempting drug target in the HIV replication cycle is reverse transcription, a step both crucial to viral replication and irrelevant to host cells[35-37]. There are several non-nucleoside inhibitors of the reverse transcriptase enzyme[33, 38, 39]. The first class, while being inhibitors of the reverse transcriptase process, achieve their activity through inhibition of virus attachment to the cells[40]. The second class interact noncompetitively with a specific allosteric binding site of the reverse transcriptase[36] However, most of the compounds are nucleoside analogues which bind competitively to the binding site of reverse transcriptase[36].

Of these inhibitors of reverse transcriptase, nucleoside analogues appear to affect a more potent and more selective action than the other classes of compounds. They do this by exerting a competitive action with respect to the normal substrates. Many 2′,3′-dideoxynucleosides[25, 26, 41, 42] and 2′,3′-substituted nucleosides[43-48] have been synthesised but the most potent of these chain terminating nucleoside analogues are 2′,3′-dideoxycytidine (ddC)[25], 3′-azido-2′,3′-dideoxythymidine (AZT)[24, 49, 50] and 2′,3′-dideoxyinosine (ddI)[51].

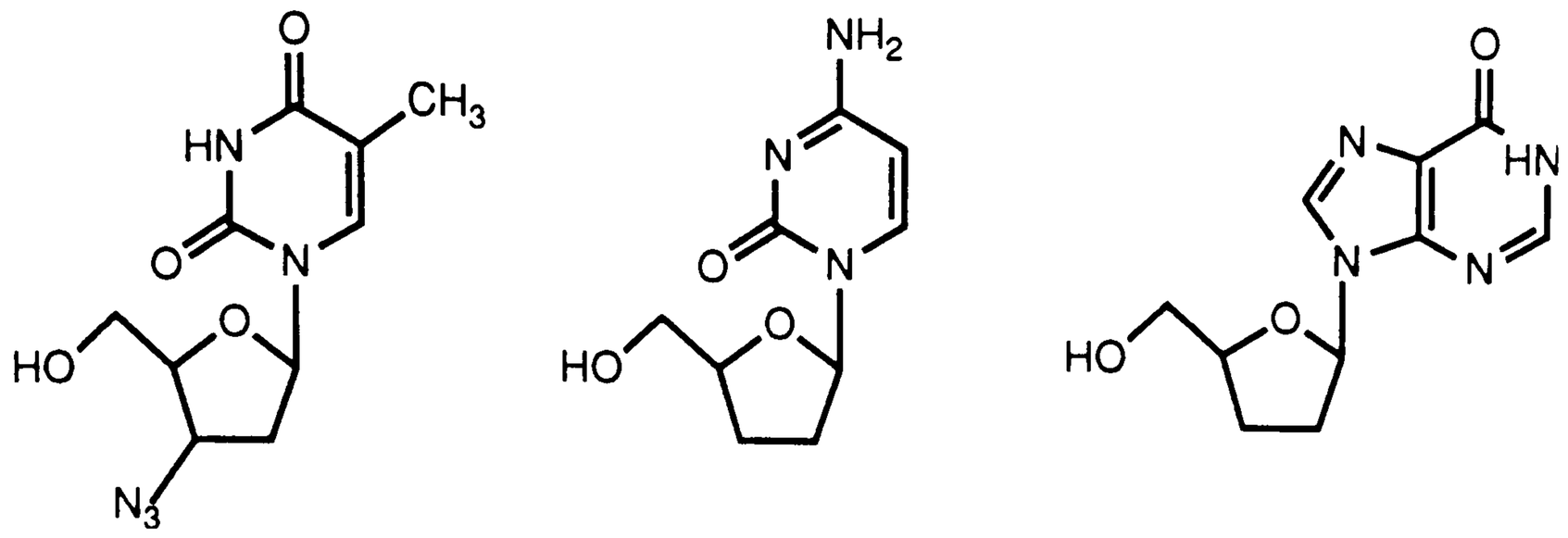


Fig. 1.4 The chemical structures of some antiviral nucleosides

The nucleosides must first be converted intracellularly to the corresponding 5′-triphosphates before any interaction with reverse transcriptase can occur. There is no difference in the ability of uninfected and virus-infected cells to phosphorylate the 2′,3′-dideoxynucleosides. Therefore, the selectivity of these nucleoside analogues must be based upon their interaction with the reverse transcriptase: to which it has an affinity 100 times greater than its affinity for cellular DNA polymerase.

The analogue is incorporated into the growing DNA chain (Fig. 1.5 B) but as the 3′-hydroxyl group of the analogue has been replaced by a hydrogen or other substituent, subsequent elongation of the DNA chain is prevented.

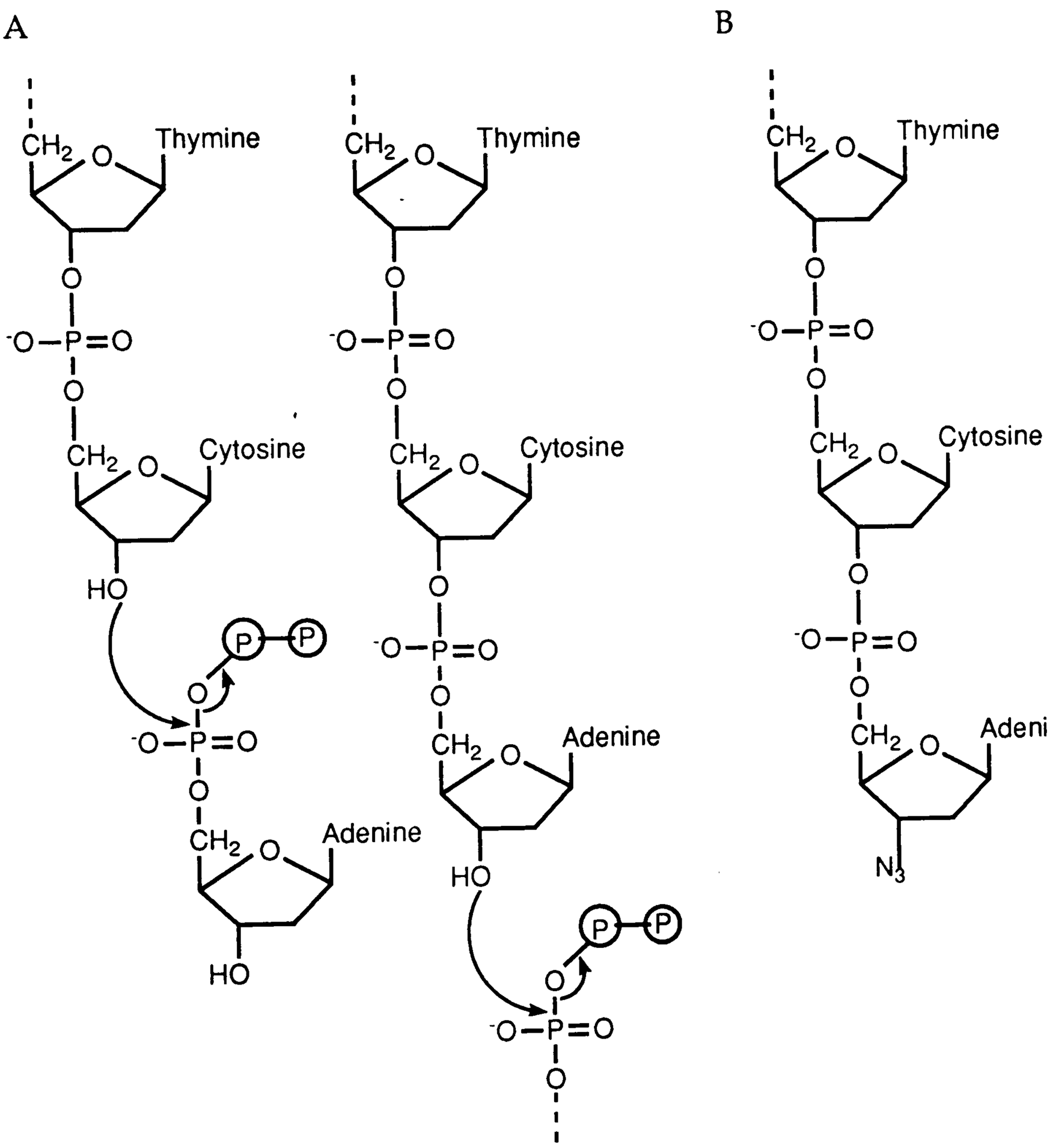


Fig. 1.5 Chain termination of the growing DNA by AZT[3, 16].

Strategies for the design of inhibitors of reverse transcriptase have been advanced greatly by the recent structural elucidation of the HIV-1 reverse transcriptase/DNA complex at 7Å resolution showing active site locations[52].

However, drug-resistant variants of HIV, resulting from mutations within the virus genome, have been recovered from patients undergoing long-term treatment with antiviral compounds[53-55]. The resistant variants arise from selective amino acid mutations in the reverse transcriptase which prevent the antiviral nucleoside analogues from binding. The analogues are therefore not incorporated into the growing chain of DNA and no chain termination occurs. This means that the need for more clinically useful therapeutic agents has heightened.

1.6: Nucleoside Synthesis

1.6.1: Chemical Synthesis

There are three main categories for the chemical synthesis of nucleoside analogues[56-60]:

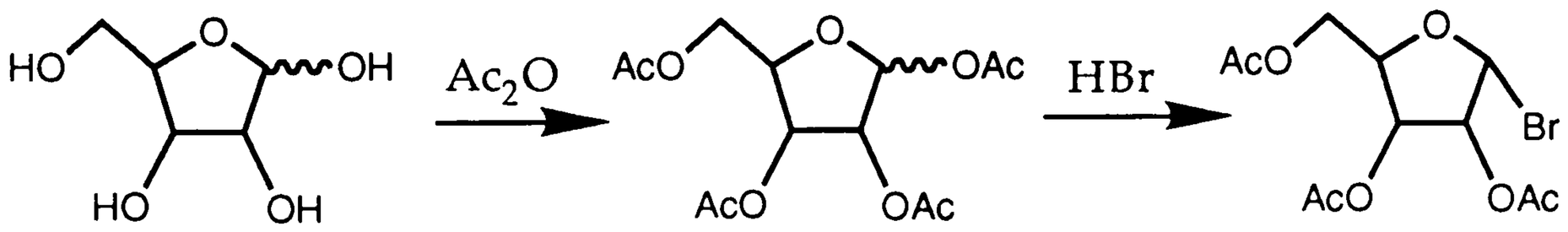
- the modification of a preformed nucleoside;
- the formation of the glycosidic bond by the fusion of a preformed base onto a sugar; or
- the *de novo* synthesis of a heterocyclic base onto a preformed nitrogen or carbon substituent at C-1 of the sugar moiety.

1.6.1.1: Formation of the Glycosidic Bond

1.6.1.1(i): Koenigs-Knorr Procedure

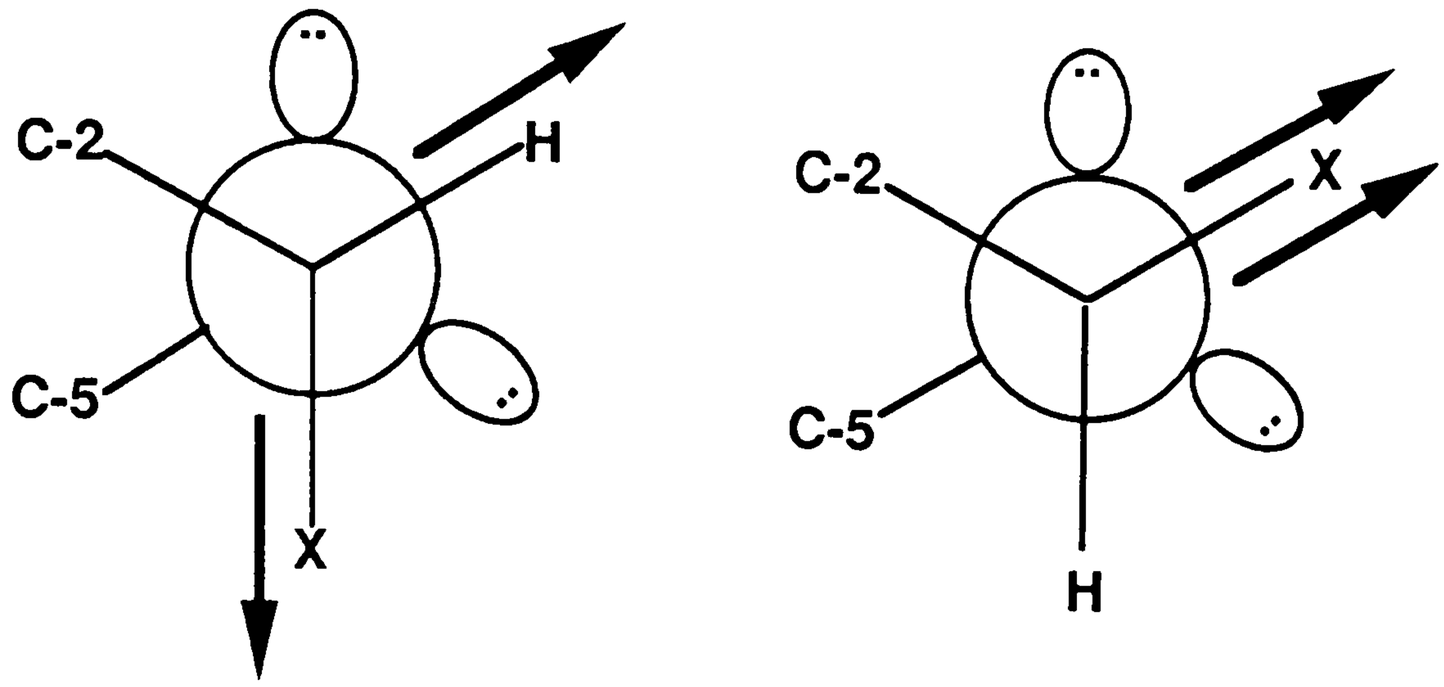
In the case of ribose sugars this reaction utilises the presence of an acyloxy-substituent at C-2 to produce selectively only the β-ribonucleosides.

The initial sugar halide is formed from the reaction of either the α- or β-D-ribofuranose tetraacetate with hydrogen bromide in acetic acid to give 2,3,5-tri-O-acetyl-α-D-ribofuranosyl bromide (Scheme 1.6).



Scheme 1.6 Synthesis of 2,3,5-tri-O-acetyl-α-D-ribofuranosyl bromide

Under these conditions the α-anomer of the bromide usually predominates because of the powerful anomeric effect. The anomeric effect is due to stereo-electronic factors arising from the more favourable dipolar interaction between the dipole associated with the ring-oxygen and the dipole associated with the substituent at C-1 in the axial position. This is shown by the Newman projection along the C-1 to O-5 bond:

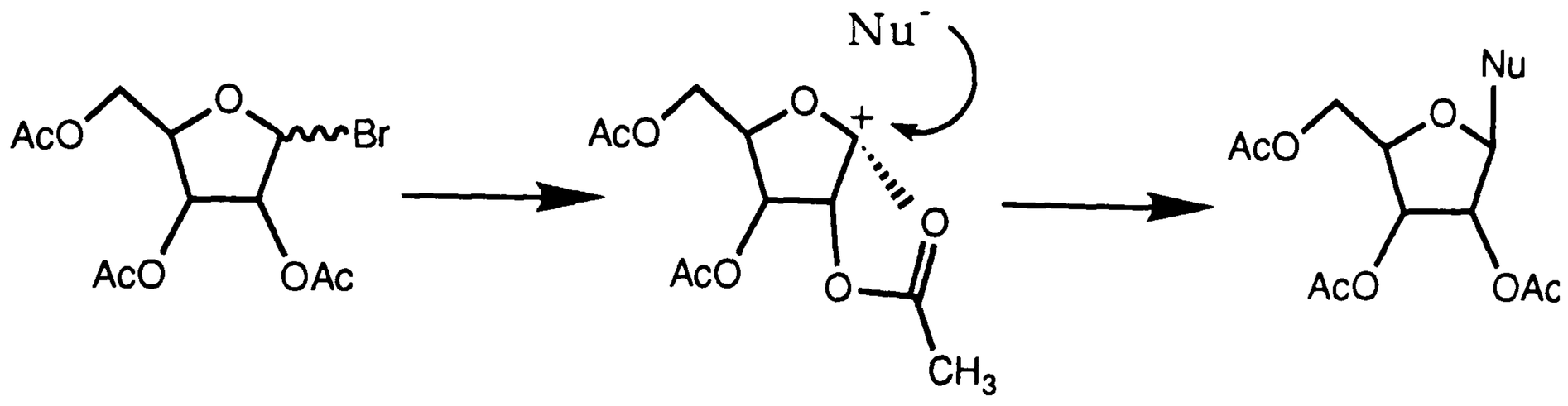


α-anomer β-anomer

Fig. 1.7 Newman projection 2,3,5-tri-O-acetyl-α-D-ribofuranosyl bromide along the C-1 to O-5 bond

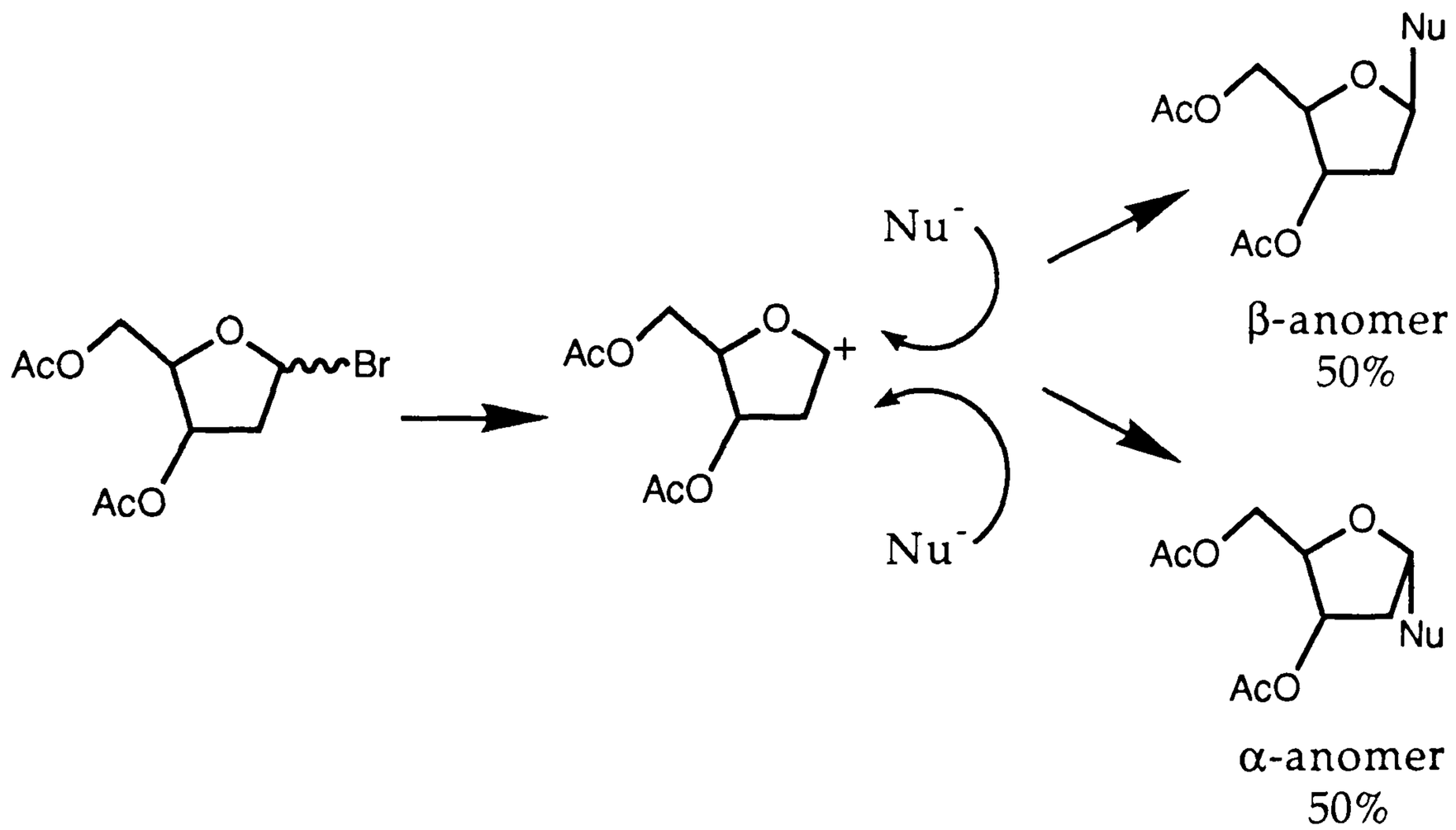
The α-anomer is thermodynamically more stable as the dipoles are not in opposition and thus represent a lower energy configuration. The extent of the anomeric effect depends upon the nature of X substituent and the solvent. Non polar solvents favour the α-anomer whereas polar solvents favour the β-anomer.

Heavy metal salts of the bases catalyse the nucleophilic displacement of the halogen substituent from the C-1 of the protected sugar to give the 1,2-trans product despite the fact that the reaction is unimolecular. The formation of only the β-product is due to the shielding of the α-face of the sugar by the neighbouring trans-acetoxyl group at C-2.



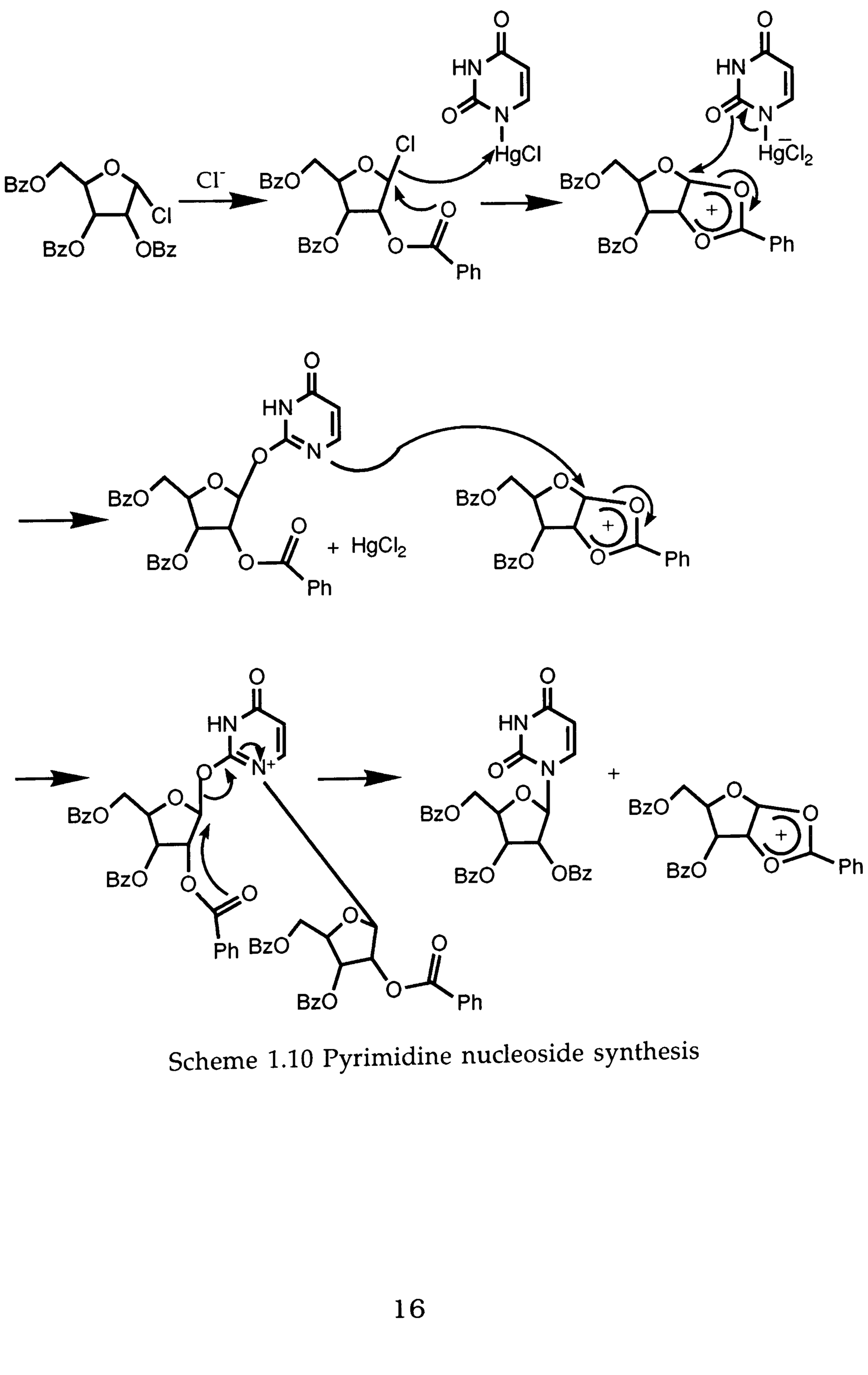
Scheme 1.8 Koenigs-Knorr displacement of halide

The stabilisation of the carbonium ion by neighbouring group participation is confirmed by the production of both α- and β-anomers when the C-2 group is lacking (Scheme 1.9).



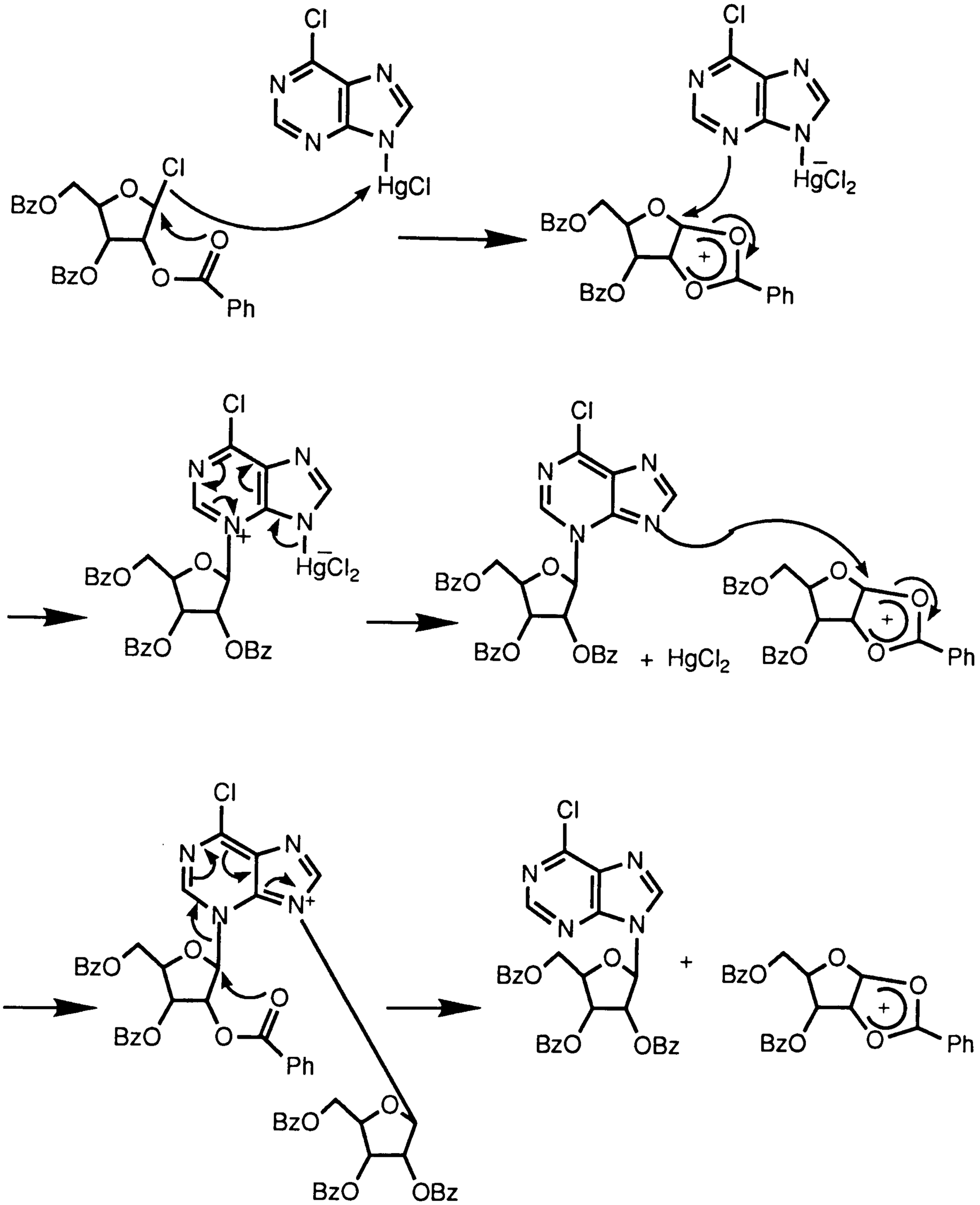
Scheme 1.9 Formation of both α- and β-anomers in the absence of a functional group at C-2

With pyrimidines, the initial product is an O-glycoside (kinetic product) which then reacts with another mole of sugar halide in the presence of mercuric chloride to form the N-glycoside (thermodynamic) product of the required β-nucleoside[58].



Scheme 1.10 Pyrimidine nucleoside synthesis

In purine nucleoside synthesis the initial N-3 glycosyl derivative undergoes an intermolecualr rearrangement to give the N-9 product.



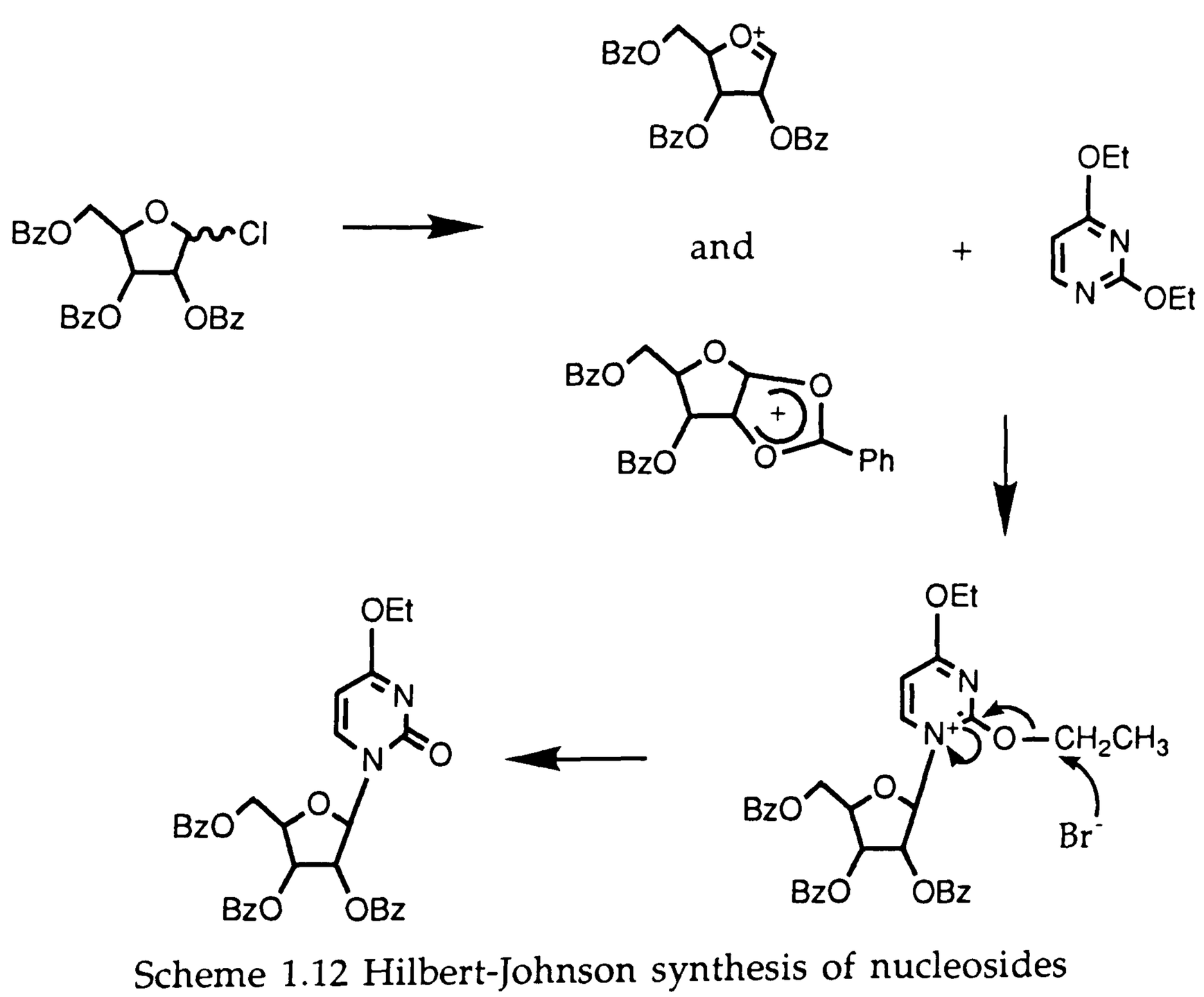
Scheme 1.11 Purine nucleoside synthesis

However, in the synthesis of 2′-deoxynucleosides and 2′,3′-dideoxynucleosides the lack of the 2′-acyl substituent means there is no stereochemical control. There is no neighbouring group participation to

protect the exo-face of the sugar moiety. This results in anomeric mixtures, so for their synthesis other methods are favoured which will be expanded on later.

1.6.1.1(ii): Hilbert-Johnson and Silyl Procedures

O-substituted pyrimidines are sufficiently nucleophilic to react directly with halogeno-sugars without any need for electrophilic catalysis. In the alkylation of a 2-alkoxypyrimidine with a halogeno-sugar the initial product is a quaternary salt which can then be reacted to give several nucleosides with different base moieties.

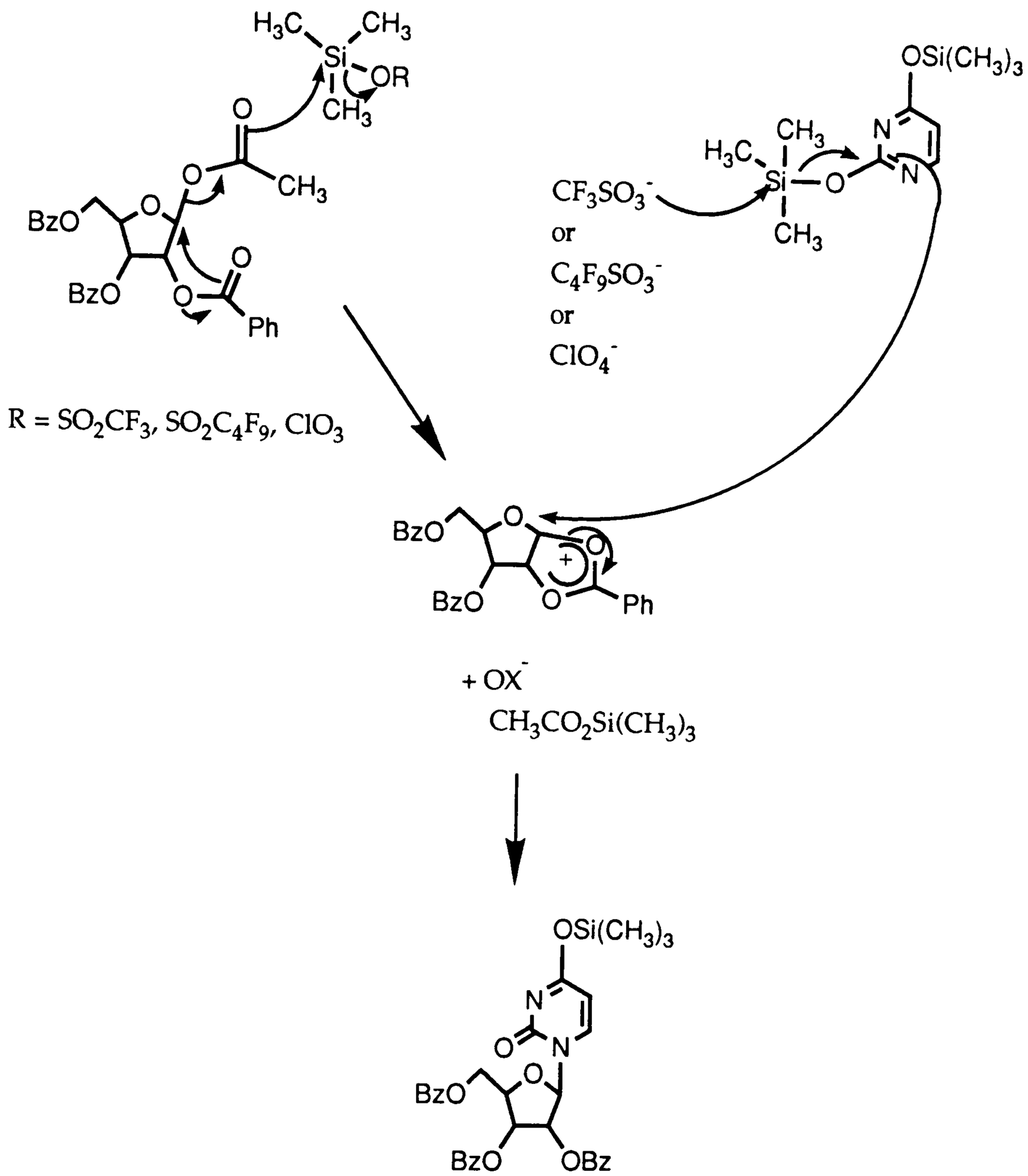


Scheme 1.12 Hilbert-Johnson synthesis of nucleosides

Owing to the mechanism of the reaction, a mixture of anomers is often produced, even with a ribose derivative bearing a 2-acyloxy-substituent. The relative contributions of carboxonium ion to the overall reaction

determines the ratio of products. In the basic reaction mechanism there is little neighbouring group participation, however, by altering the reaction conditions, such as the use of mercuric bromide, the proportion of β-anomer can be increased.

The halogenoses used in these preparations are rather unstable so the use of peracylated sugars in combination with silylated bases has the advantage of easy preparation, homogeneous reaction mixtures, and intermediate products which can be converted into a variety of nucleosides with modified bases. The use of mercuric oxide as a catalyst gave way to Lewis acid catalysts such as tin (IV) chloride and mercuric acetate, which have now been superseded by the use of silyl esters of strong acids, e.g. trimethylsilyl triflate, trimethylsilyl nonaflate or trimethylsilyl perchlorate. These Friedel-Crafts-catalysed reactions of a peracylated sugar and a silylated heterocycle in 1,2-dichloroethane, dichloromethane, or acetonitrile room temperature are fast and high yielding[61-63].



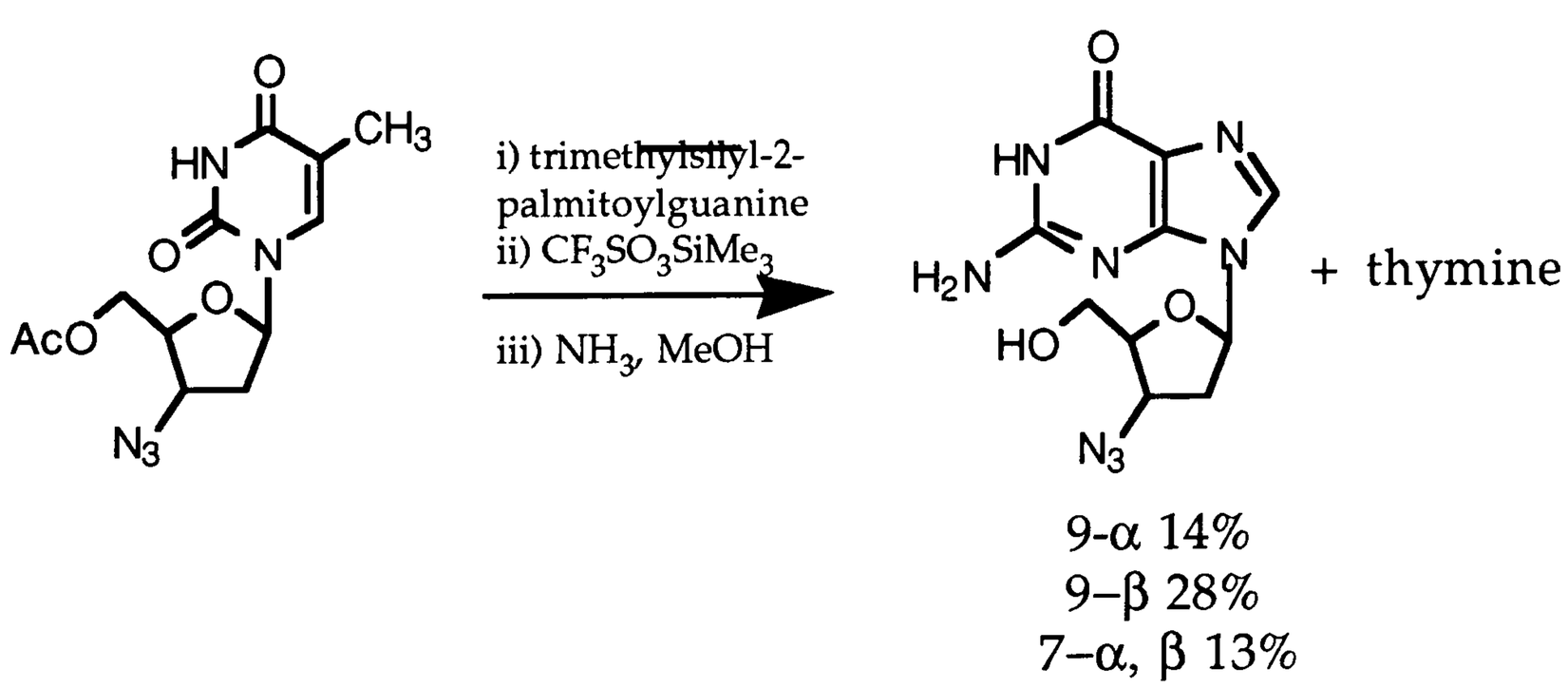
Scheme 1.13 Friedel-Crafts-catalysed nucleoside synthesis

With ribose sugars only the β-nucleoside is formed. However, some stereochemical control can be gained in the synthesis of deoxynucleosides by using suitable reaction conditions. The ratio of α and β products varies with the solvent used. A polar solvent, such as acetonitrile, will promote an S_N1 reaction mechanism which will lead to a mixture of both α and β-nucleosides. The use of a non-polar solvent, such as carbon tetrachloride, promotes the S_N2 mechanism, thus giving predominately β-nucleosides[64].

These fusion methods work well to produce a large number of nucleoside analogues, including unnatural configurations, which have modified bases and sugars that maybe difficult to prepare by other methods. The modified sugar moeity can be synthesized from a carbohydrate or noncarbohydrate precursor[65]. However, the lack of precise control of regio- and stereoselectivity and the need for protecting groups in convergent syntheses can result in substantially reduced yields.

1.6.1.2: Transglycosylation

Transglycosylation is the transfer of a sugar residue from one base to another. The reaction is particularly effective for transferring sugars from pyrimidines (which are π-deficient heterocycles) to the more basic purines (π-excessive heterocycles).

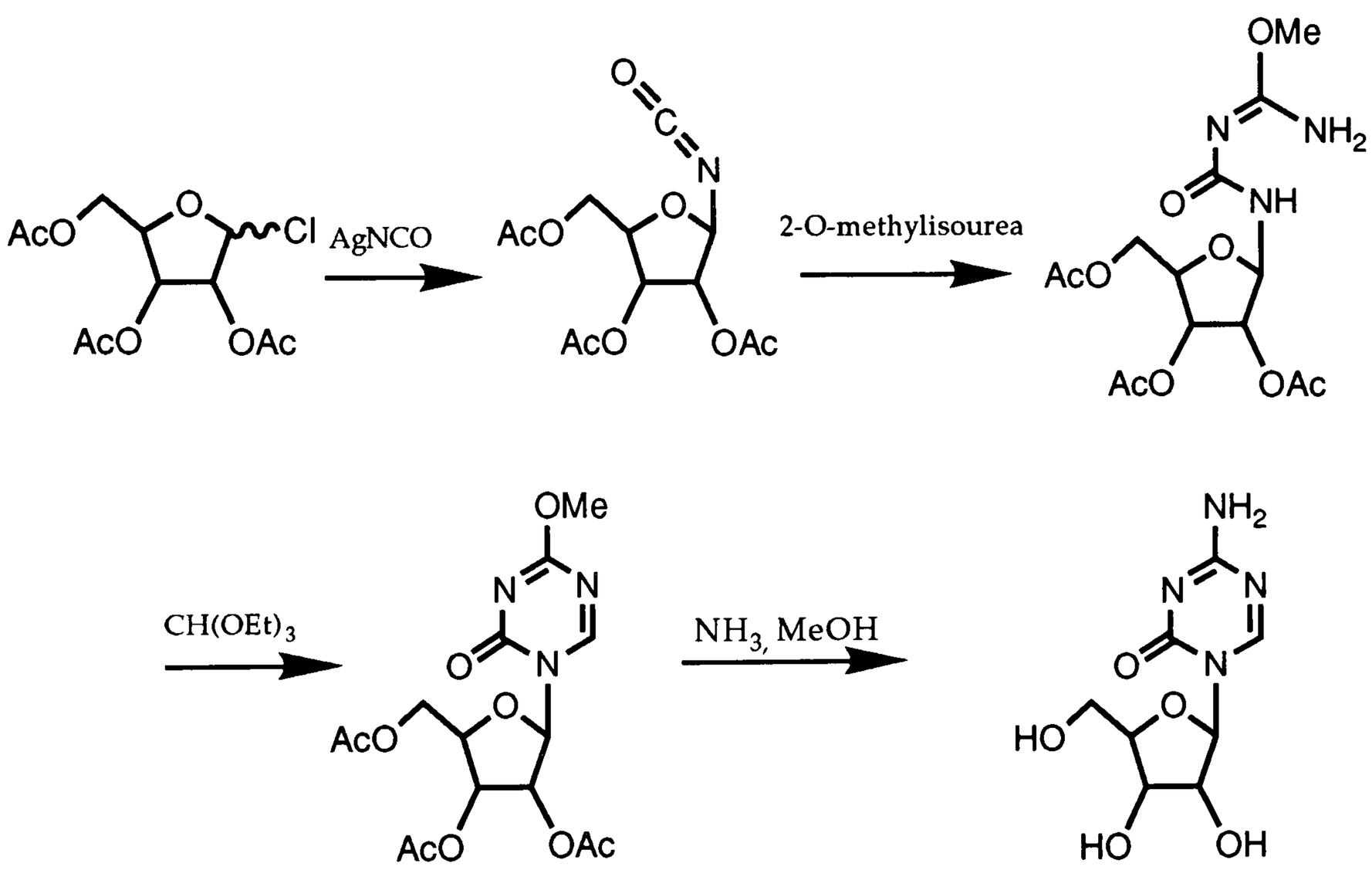


Scheme 1.14 Synthesis of nucleosides by transglycosylation

Its use in the synthesis of ribonucleoside derivatives has been demonstrated[66]. The reaction products imply an S_N1 reaction mechanism. As with other chemically catalyzed reactions, these products require chromatographic separation.

1.6.1.3: Construction of the Base onto the Sugar at C-1

The advantage of building the base onto the sugar moeity is that the position of attachment of the glycosyl residue is unambiguous and if the C-1 substituent has a β-configuration then the product nucleoside will also have this stereochemistry. There are many examples of this type of synthesis which were used historically as a method for proving structures or for the preparation of analogues (ususually aza- or deaza-nucleosides)[58].



Scheme 1.15 Synthesis of a nucleoside by constructing the base onto the sugar[67]

Nowadays, this route is often employed for the synthesis of C-nucleosides and carbocyclic nucleosides which can therefore have starting materials other than sugars or carbohydrates[56].

1.6.1.4: Modification of Naturally-Occuring Nucleosides

There are literally hundreds of reactions which can be used to transform the base of a preformed nucleoside[57, 58, 68-72] but unfortunately the reactions available for the transformation of the sugar moiety are far less common and are often multistep procedures involving protection and deprotection steps to introduce some stereo- and regioselectivity[57, 60]. Recently the interest in synthesising nucleosides with novel sugar moeities has resulted in several improved methods for the introduction of substituents into the sugar. The introduction of a fluorine substituent at various positions in the sugar moiety has produced many promising antiviral compounds. The fluorine atom is the most electronegative atom that can be introduced in an organic compound and has a van der Waals radius (1.35Å) comparable to that of hydrogen (1.17Å). Substitution of fluorine for hydrogen has a strong effect on the electronic configuration of a molecule, often reflected by a dramatic change in biological activity[73].

Presently, the most popular fluorinating agent is diethylaminosulphur trifluoride (DAST). The dialkylaminosulphur trifluorides are easy-to-handle fluorinating reagents for replacing hydroxyl and carbonyl oxygen with fluorine under very mild conditions[74]. The many different fluorinating agents and methods will be discussed in Chapter 4.

1.6.2: Enzymatic Synthesis

Many nucleoside analogues exhibiting antiviral properties are difficult to synthesise chemically owing to the presence of both modified base and sugar residues. These multistage processes are often difficult and time consuming and can produce mixtures of products in poor yields. With this in mind, chemists have been aware for a long time of the advantages that the use of enzymes as catalysts would have over purely chemical methods. Enzymes demand attention chiefly because of their efficiency, selectivity and mildness with which they catalyse reactions. For example, enzymatic glycosyl transfer reactions usually lead exclusively to the "natural" β-anomer of the nucleoside and only one site in the heterocyclic base is glycosylated[75]. The unique properties of enzymes can be exploited either, to replace existing chemical processes or in combination with chemical steps to improve existing methods.

The broad substrate specificities of some enzymes often enables reactions to be performed on analogues of natural substrates in both aqueous and non-aqueous solvents. The mild conditions of these enzyme-catalysed reactions can give good yields and are useful methods for preparing a large range of products[76-80]. However, some enzymes are strongly influenced by the concentration of substrates, products or other species present in solution which can cause problems in the scale-up for the production of large quantities of product. The problem of low solubility of some substrates and products has been addressed; for example, by the use of adenosine deaminase to convert a soluble diaminopurine nucleoside into an insoluble guanine nucleoside as the last step of a synthesis[81].

The use of immobilised enzymes means that a small amount of enzymes can be use repeatedly to produce more substantial quantities of product. Immobilisation can also enhance the thermal, mechanical, and chemical stability of the enzyme so that it becomes a recoverable catalyst. The immobilised enzyme is prevented from diffusing freely through the reaction medium by being adsorbed physically or attached chemically to a support material. Therefore, the reaction consists of two phases; the bulk solution and the immobilized enzyme with its support environment[82].

In the search for new drugs with antiviral activity many compounds have been screened. Vast effort has gone into defining and improving syntheses for nucleoside analogues with potential antiviral activity. The use of enzymes, which accept analogues as well as the natural substrates, give a convenient method for the small-scale synthesis of a huge range of highly modified base and sugar nucleoside analogues. The use of enzymes can thus be used to improve the current methods available for synthesising nucleoside analogues which are to be used in the screening programmes[78].

1.6.3: Enzymatic Synthesis of Nucleoside Analogues

1.6.3.1: Enzymatic Modification of Sugar or Base Residues

Enzymes have been used to modify existing nucleosides by selective protection and deprotection of hydroxyl groups[83-85]. The synthesis of sugar-modified nucleosides such as 2′-O-acyl esters of araA[86], and base modified nucleosides[87] have been described. By far the most useful application has been the resolution of nucleoside analogues by sugar modification[88, 89], or base modification using adenosine deaminase[81, 90].

Enzymatic resolution has also been used on intermediates in the synthesis of carbocyclic nucleosides[91, 92].

1.6.3.2: Glycosyl Transfer Reactions

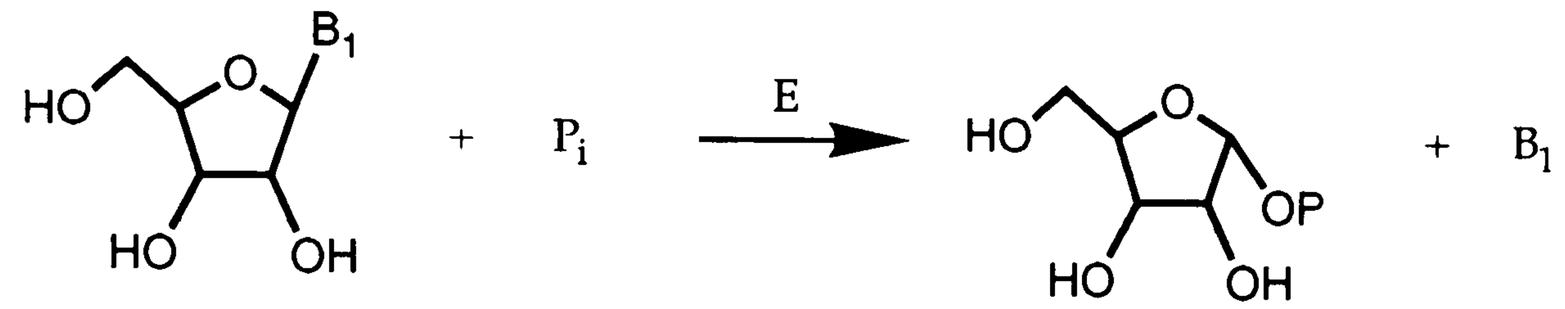
Two main classes of enzyme have been used to synthesise nucleoside analogues by transferring glycosyl residues from a donor nucleoside to an acceptor base: nucleoside phosphorylases and N-deoxyribosyltransferases[79].

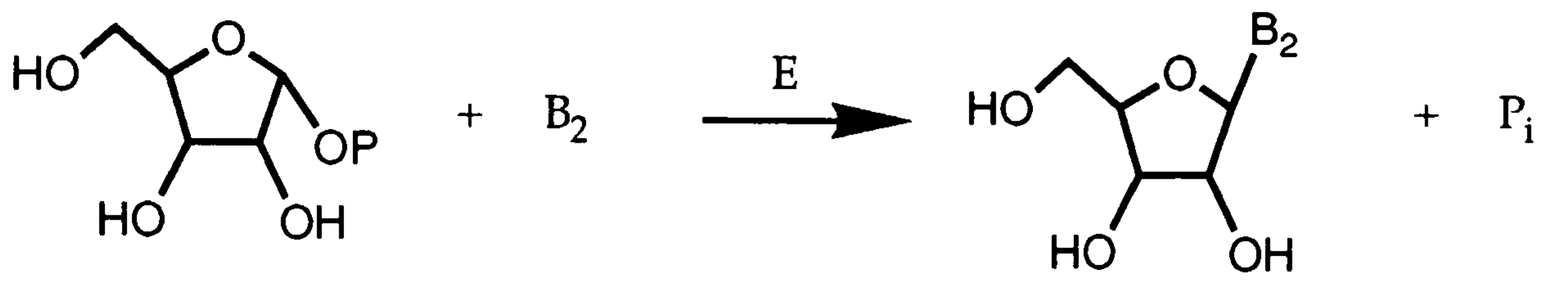
1.6.3.2(i); Nucleoside Phosphorylases

Nucleoside phosphorylases catalyse the reversible phosphorolysis of ribo- or deoxyribonucleosides affording ribose- or deoxyribose-1-phosphate and a purine or pyrimidine base. Addition of an acceptor base can lead to the formation of a new nucleoside with the equilibrium lying well in favour of nucleoside formation. Since the discovery of nucleoside phosphorylase and a general description of a mechanism[93] this method of nucleoside synthesis has been widely used. Both pyrimidine and purine nucleoside phosphorylases are known. They can be obtained from mammalian and bacterial sources and their substrate specificities can vary with the source[94]. For example, mammalian purine nucleoside phosphorylase will not accept adenosine as a natural substrate but will carry out the phosphorolysis of inosine and guanosine. However, the enzyme from *Escherichia coli* will accept all three purine nucleosides as substrates.

Two basic strategies have generally been used (with ribose as the sugar moiety in Scheme 1.16). The first involves isolation of the ribose-1-phosphate formed from a nucleoside donor and a high concentration of

phosphate. This intermediate is then used as the glycosyl donor to an added pyrimidine or purine base.





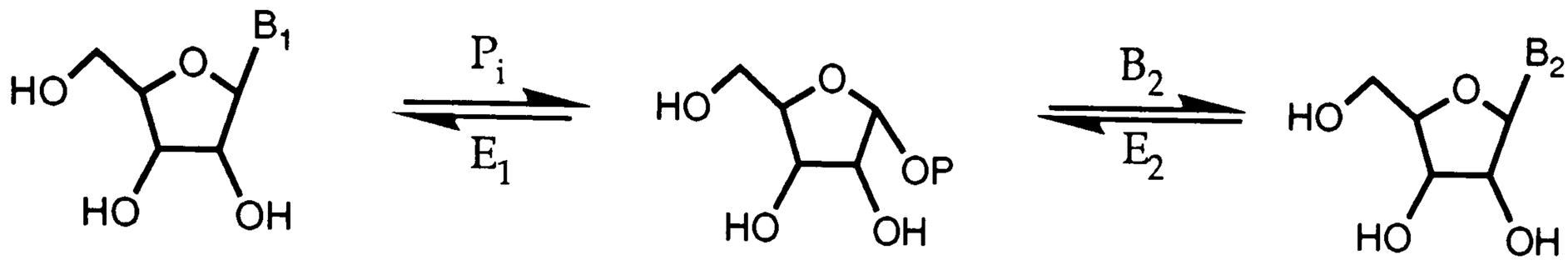
B_1= purine (or pyrimidine) base

B_2 = purine (or pyrimidine) base

E = nucleoside phosphorylase

Scheme 1.16 Two-stage synthesis of nucleosides using nucleoside phosphorylases

The second strategy involves a one-pot exchange of one base for another in the presence of a catalytic amount of inorganic phosphate without the isolation of the intermediate ribose-1-phosphate.



B_1 = pyrimidine

B_2 = purine

E_1 = pyrimidine nucleoside phosphorylase

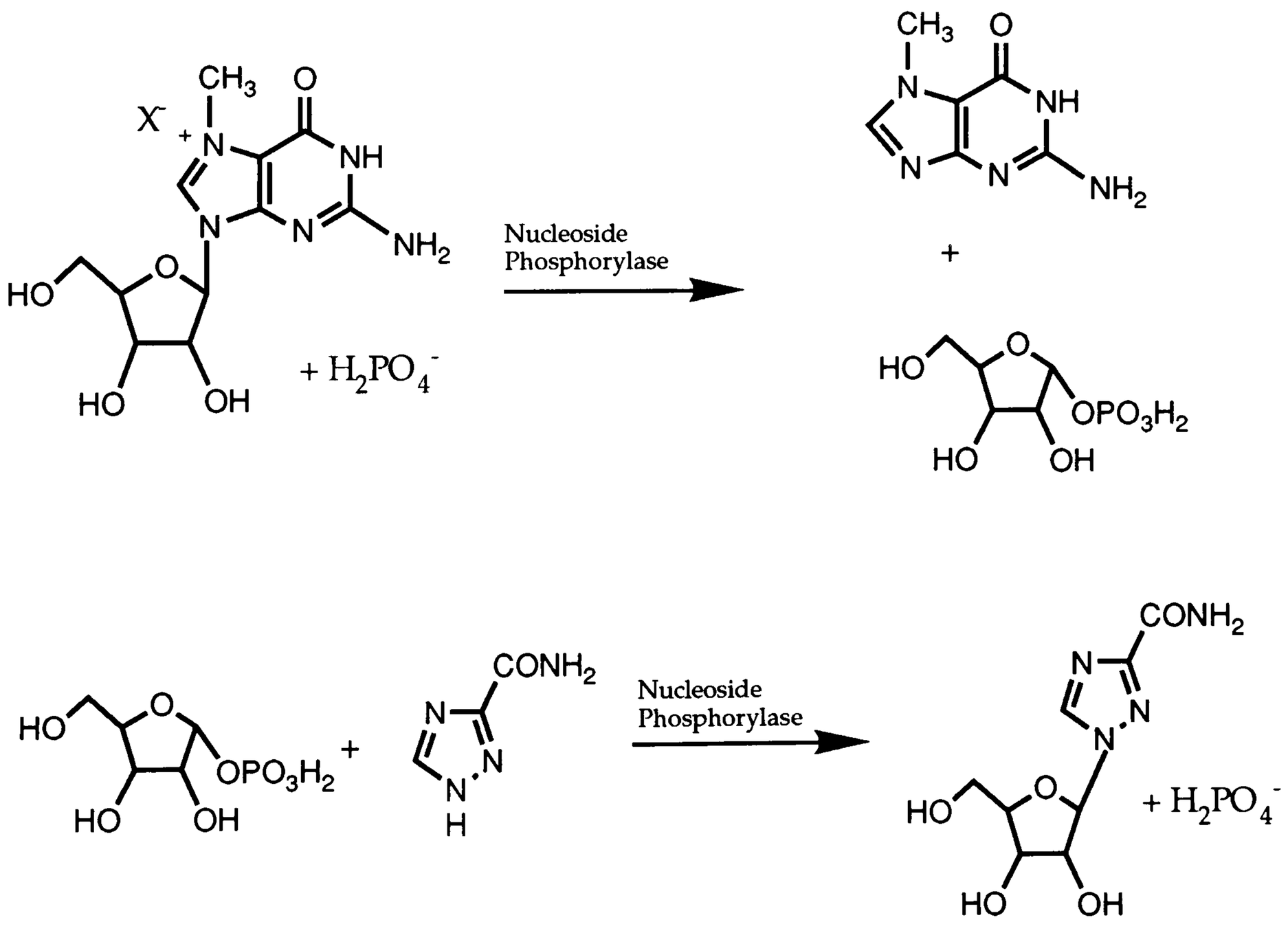
E_2 = purine nucleoside phosphorylase

Scheme 1.17 One-pot synthesis of nucleosides using nucleoside phosphorylases

Both methods have been used for the small scale production of a variety of nucleoside analogues[79]. The first strategy is the most general. The second strategy[95] has limitations such as; a) an equilibrium mixture of starting and product nucleosides can be formed from which the desired nucleoside must be separated; and b) the released base from the glycosyl donor may act as a competitive inhibitor of the enzyme and prevent the base analogue from binding. The problem of inhibition can be circumvented by using a pyrimidine nucleoside as the glycosyl donor and a purine base as the acceptor. However, both a pyrimidine nucleoside phosphorylase and a purine nucleoside phosphorylase are then required.

Both methods have disadvantages as they either require the presence of both pyrimidine and purine nucleoside phosphorylases or the isolation of the intermediate ribose-1-phosphate. These problems can be overcome and the yields of the coupled enzymatic reaction can be greatly increased by the use of an irreversible donor nucleoside[96]. By using the salt of 7-methylguanosine, as the glycosyl donor, the first stage of the

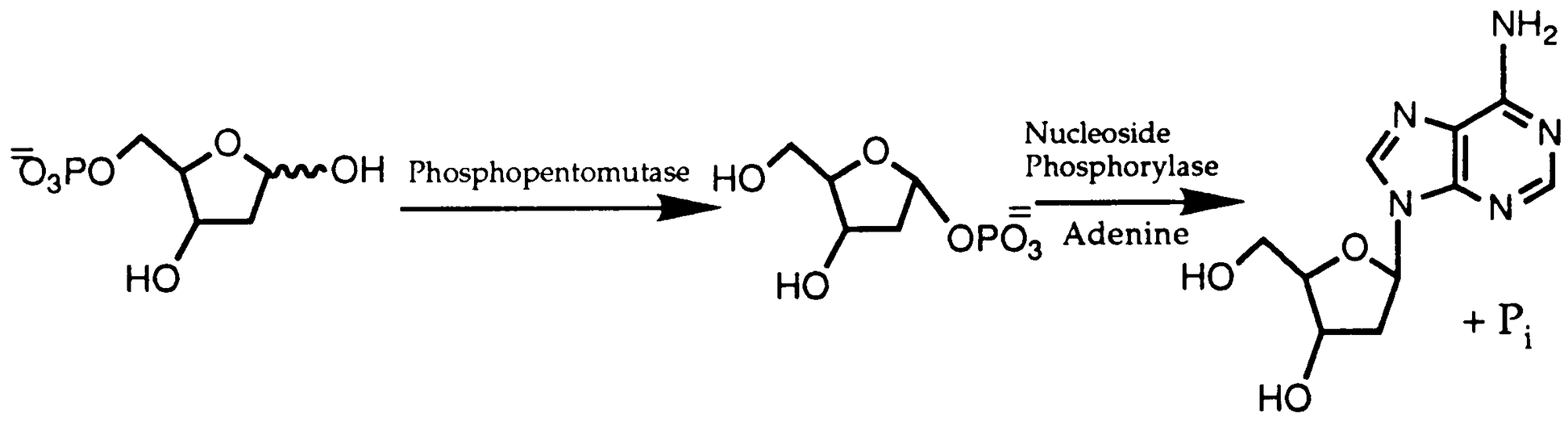
phosphorolysis reaction is made irreversible. The cleavage of the glycosyl bond produces a neutral base molecule which lacks a tautomeric proton on N-7: it would be highly unfavourable to form the positively charged nucleoside in the reverse reaction. Therefore, the 7-methylguanine does not function as a acceptor base in the reverse reaction and so the intermediate ribose-1-phosphate formed is available exclusively for the formation of product nucleoside. Only one of the nucleoside phosphorylases, not both, is required and as the 7-methylguanine is very insoluble, purification of the product nucleoside is very easy. The effectiveness of this approach was demonstrated in the synthesis of ribavarin using 1,2,4-triazole-3-carboxamide (TCA) as the acceptor base (Scheme 1.18).



Scheme 1.18 Use of 7-methylguanosine as an irreversible donor in the synthesis of nucleoside analogues

The nucleoside phosphorylases have been found to accept a wide range of nucleoside analogues and base analogues as substrates. The enzymes are able to tolerate a high degree of modification in the base component but only a limited amount of modification in the sugar moiety. So far nucleoside analogues with D-arabinofuranosyl, 2-aminoribose, 2-deoxy-3-aminoribose, and 5-deoxyribose as the sugar moiety have been synthesised. The nucleoside analogues which have been synthesised by the use of nucleoside phosphorylases have been summarised[79].

Thus, nucleoside phosphorylases have provided a regio- and stereospecific route for the synthesis of novel nucleoside analogues with potential antiviral activity. Recent advances have improved yields and purification of products. Recently, a new route has been describe which involves the coupling of nucleoside phosphorylase with phosphopentomutase[97].



Scheme 1.19 Synthesis of nucleosides using nucleoside phosphorylase and phosphopentomutase

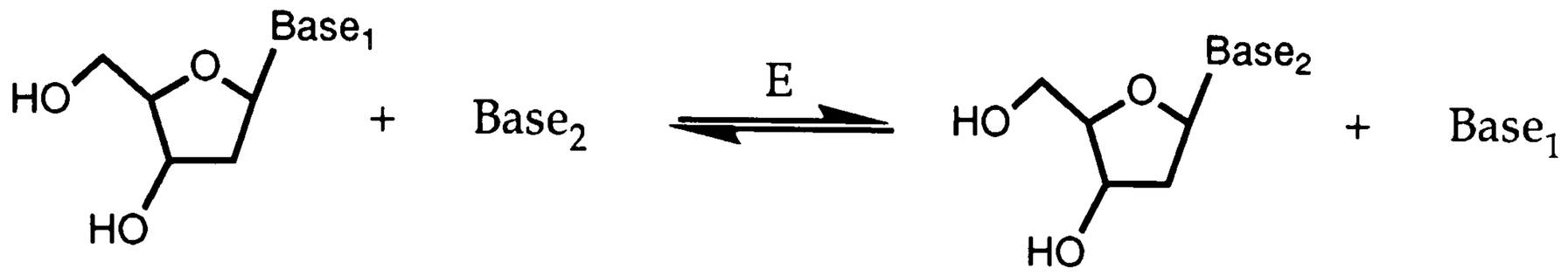
The advantage of this approach is that a pentose-5-phosphate is easier to prepare and more stable than the corresponding α-pentose-1-phosphate. This method is limited by the substrate specificity of the mutase which, to date, has been shown to accept D-ribose- and D-arabinose-5-phosphate in addition to its natural substrate 2-deoxyribose-5-phosphate. Unfortunately,

2′,3′-dideoxyribose-5-phosphate is not a substrate for the phosphopentomutase.

1.6.3.2(ii): N-Deoxyribosyltransferases

The other class of enzymes are the N-deoxyribosyltransferases which were first reported by MacNutt[98]. These enzymes were shown to catalyze deoxyribosyl transfer from 2′-deoxyribonucleosides to free purines and pyrimidines without the need for phosphate ions. The reaction involves the direct transfer of the 2-deoxyribose group from one heterocyclic base to another without the formation of a 2-deoxyribose-1-phosphate intermediate. The use of 2-deoxyribose-1-phosphate as the glycosyl donor did not result in the formation of any 2-deoxyribonucleosides. Further experiments were performed to clarify that the reaction mechanism was not the hydrolysis of the glycosidic bond followed by resynthesis with the donor heterocyclic base to give the new nucleoside[99], nor the transamination of 2′-deoxyinosine to produce 2′-deoxyadenosine[100], but was in fact trans-N-glycosylation.

Thus, the mechanism of the N-deoxyribosyltransferase was confirmed to be:



Base = purine or pyrimidine

E = N-deoxyribosyltransferase

Scheme 1.20 Reaction of N-deoxyribosyltransferase

An extensive screening has been undertaken to investigate the occurence and specificity of this enzyme[101]. Nucleoside deoxyribosyltransferases are present in appreciable levels in some organisms, notably the family *Lactobacillaceae*, which do not contain high levels of the nucleoside phosphorylase enzymes and require N-deoxyribosyltransferases to produce 2-deoxynucleosides for growth. This activity has been found in *L. helveticus*[102], *L. delbruecki*[103], *L. acidophilius*[104], and *L. lactis*[105] but is not present in extracts of *L. casei*[105], or other microbes and animals examined[106].

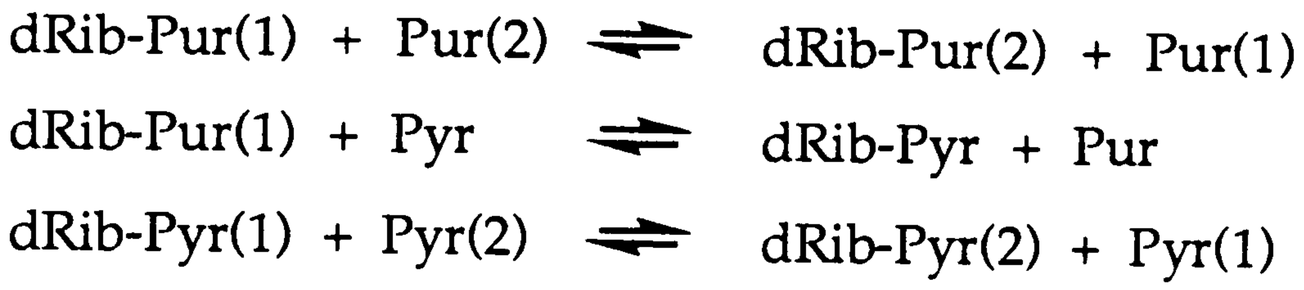
Many procedures for the purification or partial purification of the N-deoxyribosyltransferases from various lactobacilli have been reported[103-108]. The first preliminary investigations were performed on partially purified enzymes from *L. helveticus* [105] which were stable below 55°C with an optimum stability near pH 6.5, and an optimum activity near pH 5.8. However, only a few heterocyclic bases were investigated as acceptors. The workers also confirmed previous findings that inorganic phosphate

was not required for the enzymatic transfer reaction but more importantly they noted the effect of Tris buffer. The complete inhibition of transfer of the 2-deoxyribosyl group to and from pyrimidines by Tris buffer and the partial inhibition of the transfer to and from purines was indicative of the presence of at least two enzymes in the preparation.

It was shown that one enzyme, not inhibited by Tris, catalysed purine-purine transfers and the other enzyme, which was inhibited by Tris, catalysed pyrimidine-pyrimidine, pyrimidine-purine and possibly purine-purine transfers. The presence of two distinct enzymes in *L. helveticus* was confirmed by Holguin and Cardinaud[108, 109] when they purified out both activities using affinity chromatography with two types of ligand. N-deoxyribosyltransferase I catalysed the transfer of 2-deoxyribose residues exclusively between purine bases, whereas N-deoxyribosyltransferase II catalysed the transfer of 2-deoxyribose residues between purine and pyrimidine bases.

N-deoxyribosyltransferase I

N-deoxyribosyltransferase II



dRib= 2-deoxy-D-ribose

Pur = Purine

Pyr = Pyrimidine

Fig. 1.21 Activities shown by the two transferase enzymes

The two transferase types showed different physical properties and N-deoxyribosyltransferase II showed inhibition by Tris in agreement with the previous findings. Detailed kinetic studies of both transferase enzymes from *L. helveticus* were performed and the initial velocity studies supplied evidence that the 2-deoxyribosyl transfer reactions both proceed via a ping-pong bi-bi mechanism[110]. The kinetic mechanism is ping-pong because the first product is released before the second substrate combines with the enzyme and it is bi-bi because the reaction is bimolecular[111, 112].

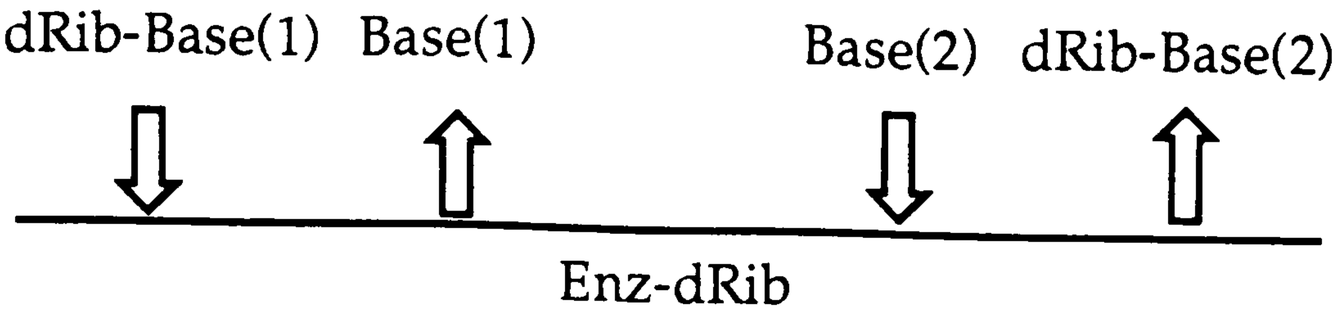


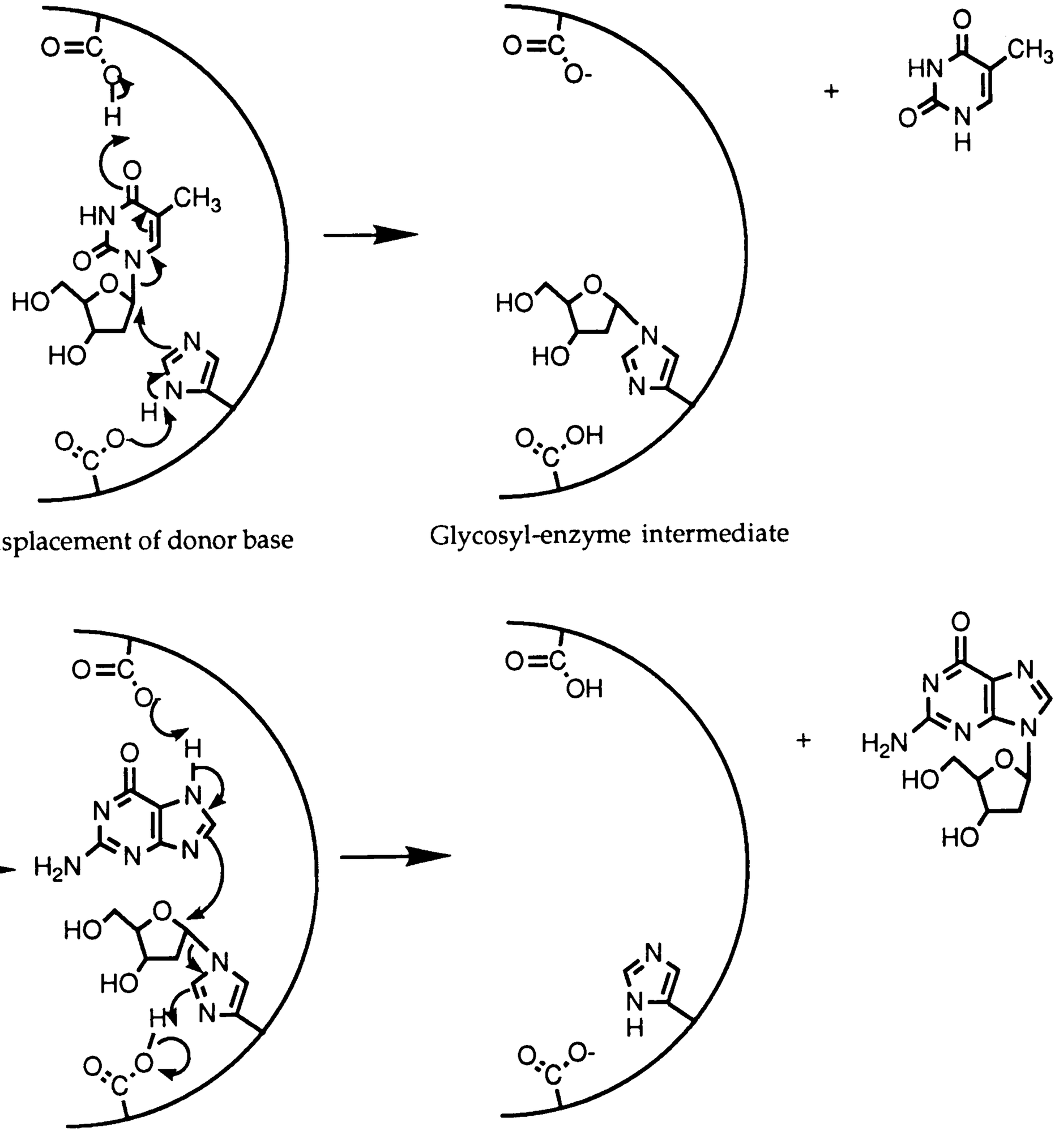
Fig. 1.22 Ping-pong-bi-bi mechanism of N-deoxyribosyltransferase

The donor nucleoside combines with the enzyme and the glycosyl bond is cleaved. The base is released, leaving a glycosyl-enzyme intermediate. The acceptor base enters the active site and combines with the glycosyl-enzyme intermediate. Finally, the new nucleoside is released, leaving the free enzyme. This mechanism postulates the existence of a stable glycosyl-enzyme intermediate in which the 2-deoxyribosyl moiety undergoes a double inversion during the course of the reaction.

A detailed study of the substrate specificities of the N-deoxyribosyltransferases from *L. helveticus* has been decribed[113]. A wide range of heterocyclic bases were accepted by the enzyme but only a very limited number of nucleosides with modified sugar residues were accepted as donors.

Recently, two distinct N-deoxyribosyltransferase activities were purified from *Lactobacillus leichmannii* and their substrate specificities investigated[114]. As with enzymes from *Lactobacillus helveticus,* these enzymes were capable of accepting a wide number of heterocyclic base analogues as substrates but very little tolerance was shown towards donor nucleosides with modified sugar residues. Once again the transfer reaction proceeded via a ping-pong bi-bi mechanism and kinetic and radiolabelling experiments provided preliminary support for the existence of a covalent glycosyl intermediate. Chemical modification of N-deoxyribosyltransferase

II with specific chemical reagents suggested that histidine and/or carboxyl residues may participate in binding and catalysis at the active site of the enzyme. A proposed mechanism was suggested which fits with the information ascertained so far and highlights the double-inversion of the 2-deoxyribosyl moiety to produce β–nucleosides exclusively.



Scheme 1.23 Proposed mechanism of N-deoxyribosyltransferase

N-deoxyribosyltransferase has also been purified and characterised from *Leuconostoc mesenteroides* subspecies *cremoris*[115]. A single multifunctional enzyme capable of carrying out the transfer of the 2-deoxyribosyl moiety from either pyrimidine or purine nucleosides to either pyrimidine or purine bases was found to be present in this strain of *Leuconostoc*. Kinetic studies carried out on the purified enzyme showed that the transfer reaction also proceeded via a ping-pong bi-bi mechanism. Further evidence for the existence of a glycosyl-enzyme intermediate was provided by radiolabelling studies

Microcrystals of nucleoside deoxyribosyltransferase obtained from *Lactobacillus helveticus* have been reported[102], but these were not of sufficient quality to be used for X-ray studies. However, crystals of recombinant bacterial N-deoxyribosyltransferase have been grown from solutions of ammonium sulphate[116]. Molecular weight studies performed on the purified protein revealed a molecular weight for the intact enzyme of approximately 110,000 and a subunit molecular weight of approximately 18,000. Therefore, the enzyme is a hexamer of six identical subunits. These studies were only performed on one of the transferase activites but no indication as to which one, was given. Only a very brief paper was published on this work; the complete data is yet to be published.

1.7: Function of N-Deoxyribosyltransferase Activity

Within cells, nucleosides can be synthesised in two ways: the *de novo* synthesis; or the salvage pathway. In the *de novo* pathway, the purine and pyrimidine bases are constructed onto the ribose moiety in several steps which is an overall high energy process. A much more efficient method is the salvaging of bases produced by hydrolysis of nucleic acids and

nucleotides. The metabolic function of the N-deoxyribosytransferase is believed to be part of nucleic acid synthesis[117]. The effects of varying vitamin B_{12}, 2′-deoxyribonucleoside, or free purine and pyrimidine bases have been investigated and showed that vitamin B_{12} participates in the reduction of ribonucleosides to 2′-deoxyribonucleosides[118-120]. In the absence of vitamin B_{12} a single 2′-deoxyribonucleoside is converted to the full complement of DNA precursors by the N-deoxyribosyltransferase catalysed transfer of the 2-deoxyribose residue to the exogenous free bases[121, 122]. In 2′-deoxyribonucleoside- or vitamin B_{12}-limiting cultures, filamentous forms of *Lactobacillus leichmannii* occur as a result of unbalanced growth due to impaired DNA synthesis.

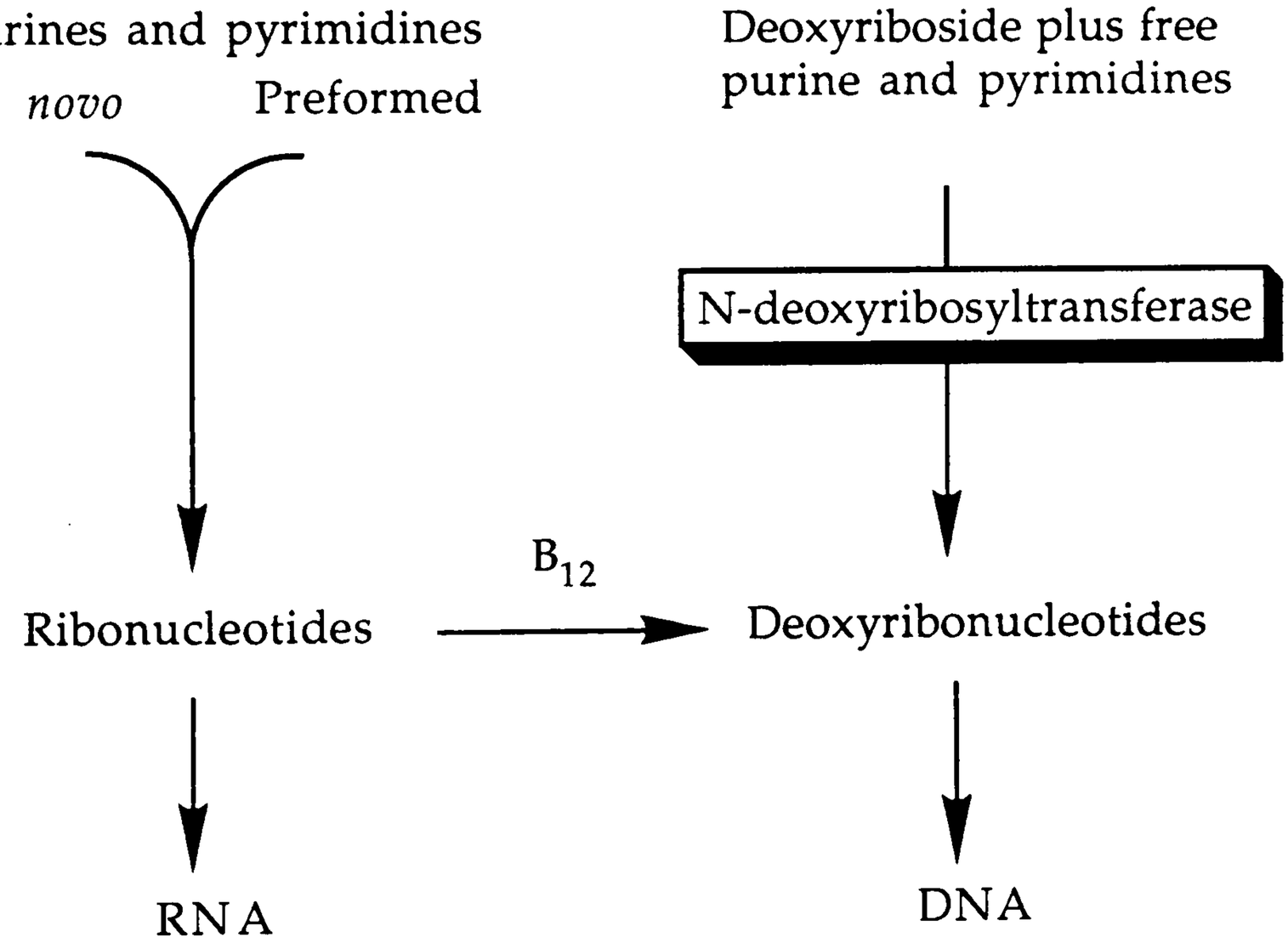


Fig. 1.24 Function of N-deoxyribosyltransferase in cells

Thus, the N-deoxyribosyltransferase activity serves as an alternative pathway for 2′-deoxynucleotide synthesis in the absence of vitamin B_{12}.

1.7.1: N-Deoxyribosyltransferases in the Synthesis of Nucleoside Analogues

The N-deoxyribosyltransferases offer an alternative route to nucleoside phosphorylases for the synthesis of nucleoside analogues[78]. For the synthetic preparation of nucleosides with possible antiviral activity, the transferases from *Lactobacillus helveticus* and *Lactobacillus leichmannii* are usually used as crude preparations without the separation of the two discrete transferase activities[123, 124]. This is a very convenient method as the minimum of purification of the transferase enzyme is required. As with the nucleoside phosphorylases, the N-deoxyribosyltransferase reaction is a very regio- and stereoselective process with the glycosyl bond being formed at only one nitrogen within the heterocyclic base in a β-configuration[125].

The use of crude enzyme preparations can simplify the synthetic procedure greatly, but degradation of products and substrates by contaminating hydrolytic and/or deaminating activities in the crude enzyme preparation can occur when long reaction times are used. The addition of water-miscible organic solvents to the synthetic reactions catalysed by the crude enzyme preparations can inhibit these degradative side reactions. It was found that the glycosyl transfer reaction was not affected provided that the concentration of the organic solvent was kept low. However, the best results were achieved by the addition of 10% (v/v) ethylene glycol to the reaction which inhibited any contaminating enzyme while allowing the transfer reaction to proceed as normal[126].

In the transferase reactions, thymidine or 2′-deoxycytidine are the most effective glycosyl donors. The transferases will only accept 2′-deoxy-, 2′,3′-dideoxy-, and 2′,5′-dideoxynucleosides as the donor nucleosides: no other sugar modifications have yet been found which are tolerated. However, considerable structural variation is tolerated in the acceptor bases, as was shown by the synthesis of a series of 2′-deoxyadenosine derivatives[127].

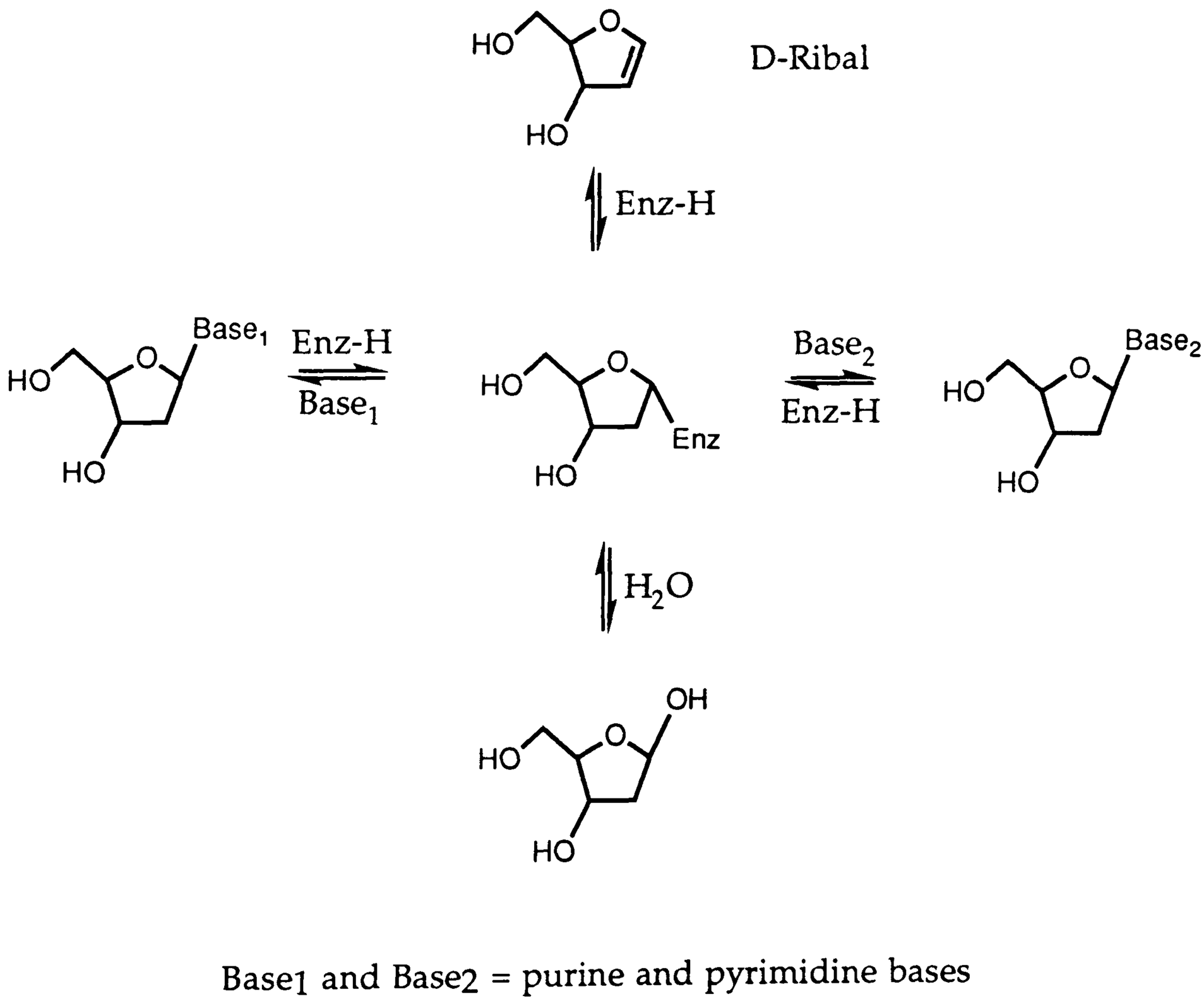
Initially the transferases were used to synthesise 2′-deoxynucleoside analogues with therapeutic activity, such as 2-chloro-2′-deoxyadenosine which has antileukemic and immunosuppressive activity[128, 129], and imidazole-2′-deoxynucleosides[75]. The reaction is ideal for the preparation of radioactively labelled compounds as the radiolabel is found exclusively in either the base or the sugar moiety of the product nucleoside and there is no contamination in other parts of the nucleoside[130]. This has been shown in the synthesis of 2-[^{14}C]-2′-deoxyribofuranosyl-5-trifluoromethyl uracil[131].

It has been found that the efficacy of the potent antiviral drugs 2′,3′-dideoxyadenosine and 2′,3′-dideoxyinosine are diminished inside cells by attack from adenine deaminase and mammalian PNPase respectively[25]. However, 2-halo-2′,3′-dideoxyadenosine derivatives have been found to be resistant to this catabolism and have been synthesised by an initial enzymatic transfer reaction to yield the corresponding 2-halo-2′-deoxyadenosine, followed by a Barton elimination of the 3′-hydroxyl group to yield the required 2-halo-2′,3′-dideoxyadenosine[132].

More recently the transferases have been used in the synthesis of 2′,3′-dideoxynucleosides which have been found to have antiviral activity[130]. A vast range of base modified 2′,3′-dideoxynucleosides have now been

synthesised by enzymatic means for screening as potential anti-HIV drugs[133-137].

A very interesting discovery is the production of 1,4-anhydro-2-deoxy-D-erythro-pent-1-enitol (D-ribal), a glycal of ribose, in the enzymatic reactions. In the absence of acceptor bases, the N-deoxyribosyltransferase catalyses the slow hydrolysis of the 2′-deoxynucleosides, to generate spontaneously D-ribal which only disappears later as 2′-deoxynucleoside hydroysis approaches completion[138].



Base1 and Base2 = purine and pyrimidine bases

Enz-H = a nucleophilic group at the enzymes's active site

Scheme 1.25 Mechanism of nucleoside and D-ribal formation by N-deoxyribosyltransferase

It has been found that in the absence of heterocyclic bases the N-deoxyribosyltransferase catalyses the hydration of D-ribal but when bases are present it catalyses the synthesis of 2′-deoxyribonucleosides. This affords a new method for the preparation of 2′-deoxynucleosides, but as yet only natural substrates have been reported. This reaction also circumvents the need to separate the product nucleoside from any substrate nucleoside and base that remain at the end of the reaction, by eliminating the substrate nucleoside from the reaction mixture. The stereochemistry of the nucleoside formation from D-ribal also supports previous evidence for the presence of a deoxyribosyl-enzyme intermediate.

In summary, the N-deoxyribosyltransferases have practical application in the synthesis of a wide range of nucleoside analogues. The transferases are able to tolerate a minor amount of modification in the sugar moiety and a large amount of modification in the base moiety. The N-deoxyribosyltransferases have several advantages over the nucleoside phosphorylases which improve the enzymatic synthesis of nucleosides, such as the fact that, only one enzyme is required for the transfer of the glycosyl residue between purines and pyrimidines in the synthesis of a whole range of analogues, thus simplifying the procedure and giving a cleaner reaction.

Further studies on the N-deoxyribosyltransferases could help to optimise the reaction conditions. Information about the active site and the mechanism of the transfer reaction would be useful in the understanding of the glycosyl transfer reaction for the directed chemical synthesis of possible substrates. The substrate specificity of the enzyme could be investigated to the full to try to find novel nucleoside donors with either

modified sugars for the synthesis of a whole range of new nucleoside analogues or modified bases to act as improved or irreversible donors.

CHAPTER 2

ENZYMATIC SYNTHESIS OF NUCLEOSIDES

2.1: Background

Considerable attention has been focussed on structure-activity correlations of 2′,3′-dideoxynucleoside analogues as potential anti-HIV drugs[139]. The 2′,3′-dideoxynucleosides of purines and pyrimidines have been shown to be the most active anti-HIV nucleosides. Therefore, much effort has been invested in the synthesis of these types of compound.

Some nucleoside analogues which, when converted to their 5′-triphosphates, act as chain terminators of DNA synthesis, can be effective inhibitors of the replication of HIV both *in vitro* and *in vivo*. Recently, toxicity problems have arisen in the clinical use of some nucleoside analogues, for example, treatment with AZT can cause bone marrow suppression leading to anaemia and neutropenia[140]. Thus, the synthesis of novel nucleoside analogues which have anti-HIV activity and which show low toxicity is of interest.

There are numerous chemical routes to synthesise 2′,3′-dideoxynucleosides by either modification of an existing nucleoside or fusion of a sugar and base to form the nucleoside. The modification of existing nucleosides includes: the reduction of 3′-deoxy-3′-halogenonucleosides[141]; the radical-initiated reduction of thiocarbonates to an olefin[142-144]; the formation of a cyclonucleoside which is ring-opened to an olefin[145, 146]; and the reductive deoxygenation of vicinal diols to an

olefin[147]. In the last three cases the olefins are then reduced to yield the 2′,3′-dideoxynucleosides.

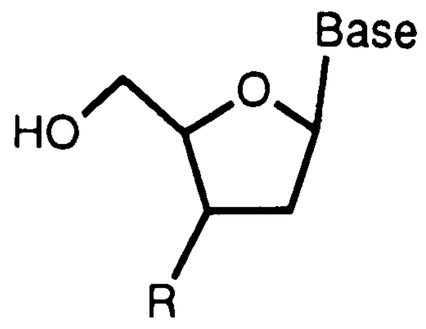
Chemical approaches have limitations as to the type of nucleosides that can be synthesised. In contrast, the convergent synthesis enables the synthesis of a large number of nucleoside analogues with structural variations in both the sugar moiety and the base along with unnatural configurations[65]. The 2,3-dideoxyribose intermediate can be synthesised from a carbohydrate or non-carbohydrate precursor. The heterocyclic base, synthesised as a separate entity, is then coupled to the sugar to yield the 2′,3′-dideoxynucleoside.

Both these synthetic procedures are not without difficulties. Therefore, much interest has been shown in the development of enzymatic methods for the synthesis of nucleoside analogues where precise stereo- and regiochemical control is possible without the use of protecting groups on reactive residues. N-Deoxyribosyltransferases are specific and glycosylate only one nitrogen in a heterocyclic base to give a nucleoside with a β-configuration. Several enzymatic synthesise of nucleoside analogues have been described[75, 125, 127, 133, 134] using a crude nucleoside N-deoxyribosyltransferase from *Lactobacillus leichmannii*. This route is complementary to one involving a combination of purine and pyrimidine phosphorylases[148]. The availability of enzymatic synthetic methods means that a range of novel nucleosides is now accessible.

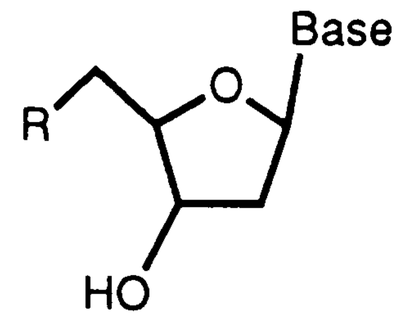
2.1.1: N-Deoxyribosyltransferases from *Lactobacillus leichmannii*

Lactobacillus leichmannii is a member of the subdivision of obligately homofermentative lactobacilli which ferment glucose almost entirely to lactic acid. These lactic acid bacteria occur as regular, non-sporing, gram-positive rods with rounded ends (0.5–0.8 x 2–9μm). The cells of *Lactobacillus leichmannii* occur singly, in pairs and in short chains, and can be isolated from milk, cheese, compressed yeast and grain mash. They require not only carbohydrates as energy and carbon sources but also nucleotides, amino acids and vitamins are essential growth factor requirements[149]. Both N-deoxyribosyltransferase I and N-deoxyribosyltransferase II have been purified from *Lactobacillus leichmannii*[114], but for the synthetic reactions a crude preparation of the enzyme is employed. The crude preparation contains both transferase activities as well as many other enzymes, such as hydrolases and deaminases. The other enzyme activities are inhibited by the addition of 10% ethylene glycol to the reaction mixture[126]. The addition of the organic solvent protects the products of the reaction from degradation by other enzymes present in the crude preparation. This improves the yields of products and removes the necessity of purifying the N-deoxyribosyltransferases, thus greatly simplifying the procedure.

It has been shown that a wide range of bases act as competent acceptors with N-deoxyribosyltransferases but only very minor modifications on the sugar moiety are tolerated[114]. Analogues that lack a hydroxyl group in the 3′- or 5′-position are accepted by the enzyme as glycosyl donors[109, 134, 150]. However, the rate of transfer of the 2,3-dideoxyribosyl moeity occurs at a much slower rate than the natural 2-deoxyribosyl substrate[130].



R = H, OH



R = H, OH, F, Cl, I

Fig. 2.1 Sugar moeities accepted as glycosyl donors by N-deoxyribosyltransferase

With this in mind it was decided to use N-deoxyribosyltransferase to investigate new synthetic routes to 2′,3′-dideoxynucleosides and to synthesise 2′,3′-dideoxynucleosides with modified bases as possible anti-HIV compounds.

2.2: RESULTS AND DISCUSSION

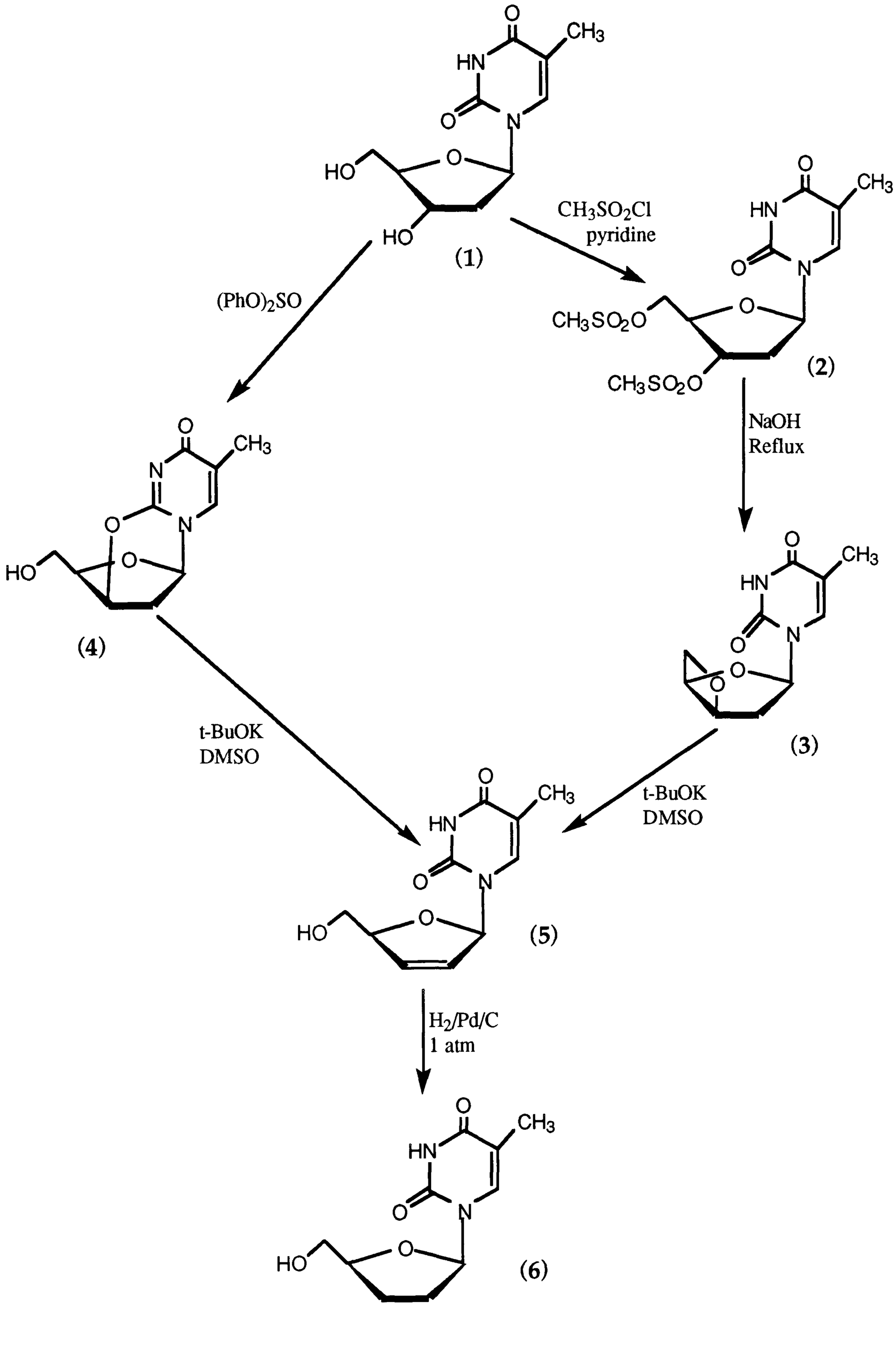
2.2.1: Chemical Synthesis of Nucleosides and Bases

Synthesis of Glycosyl Donors

For the synthesis of 2′-deoxy- and 2′,3′-dideoxynucleosides, suitable glycosyl donors are required which give high levels of transfer with a large number of acceptor bases. From substrate specificity studies performed it has been shown that 2′-deoxycytidine is a better 2-deoxyribosyl donor than thymidine or 2′-deoxyuridine[105] and this is also true of the corresponding 2′,3′-dideoxyribosyl donors. These glycosyl donors can all be purchased from Sigma: 2′-deoxycytidine (£17.60 per 1g), thymidine (£6.20 per 1g), 2′,3′-dideoxycytidine (£320.20 per 1g), and 3′-deoxythymidine (£1757.00 per 1g). However, due to the expense it was necessary initially to chemically synthesise the 2′,3′-dideoxynucleosides which were to be used as the 2,3-dideoxyribosyl donors.

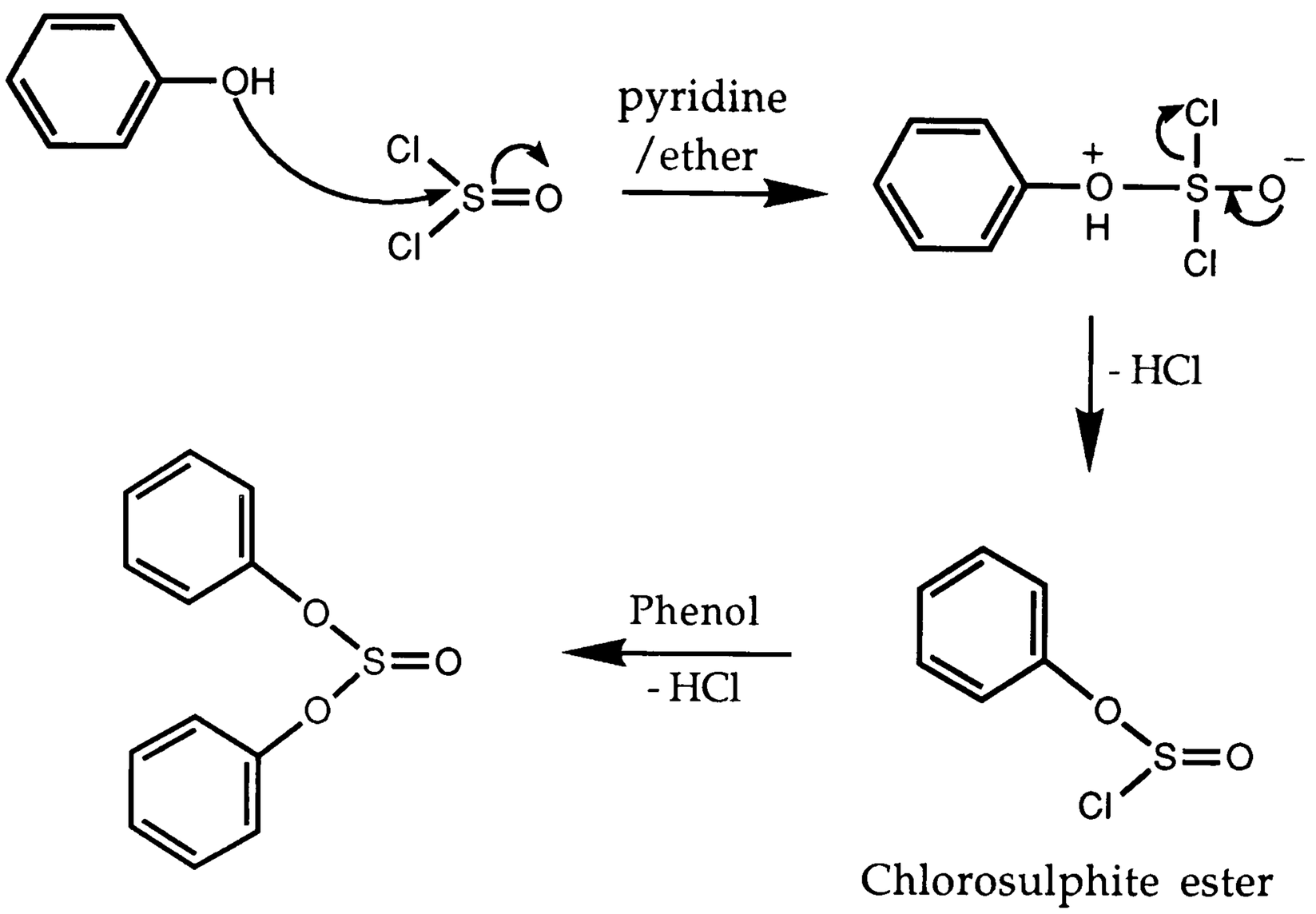
2.2.1.1: Synthesis of 3′-deoxythymidine (6)

There have been many papers published on the synthesis of 1-(2,3-dideoxy-β-D-glycero-pento-2-enofuranosyl)thymine (**5**) and the various intermediates which can be combined to form a useful synthesis of 3′-deoxythymidine (**6**)[146, 151]. Two different routes were employed, via cyclonucleosides, to synthesise 1-(2,3-dideoxy-β-D-glycero-pento-2-enofuranosyl)thymine (**5**) which after hydrogenation gave 3′-deoxythymidine (**6**).



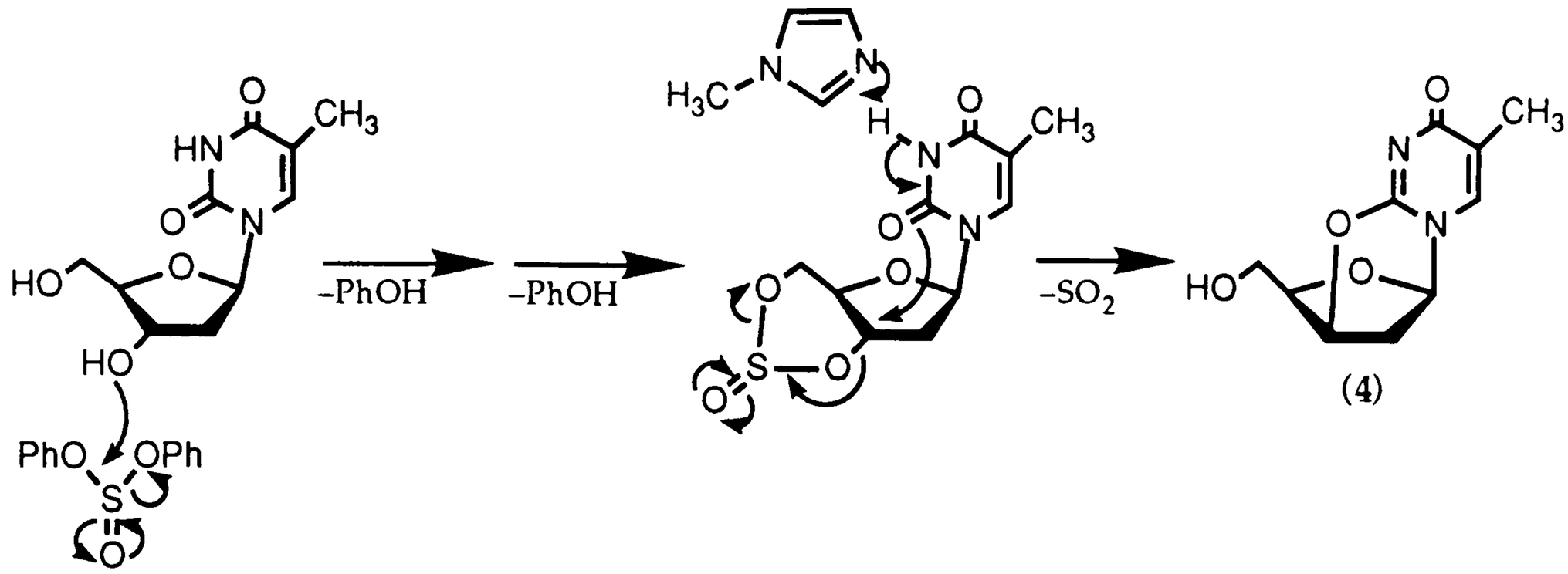
Scheme 2.2 Total synthesis of 3′-deoxythymidine (**6**)

In order to synthesise the 2,3′,-anhydrothymidine (4) intermediate it was necessary to synthesise diphenyl sulphite from phenol and thionyl chloride[152].



Scheme 2.3 Synthesis of diphenyl sulphite

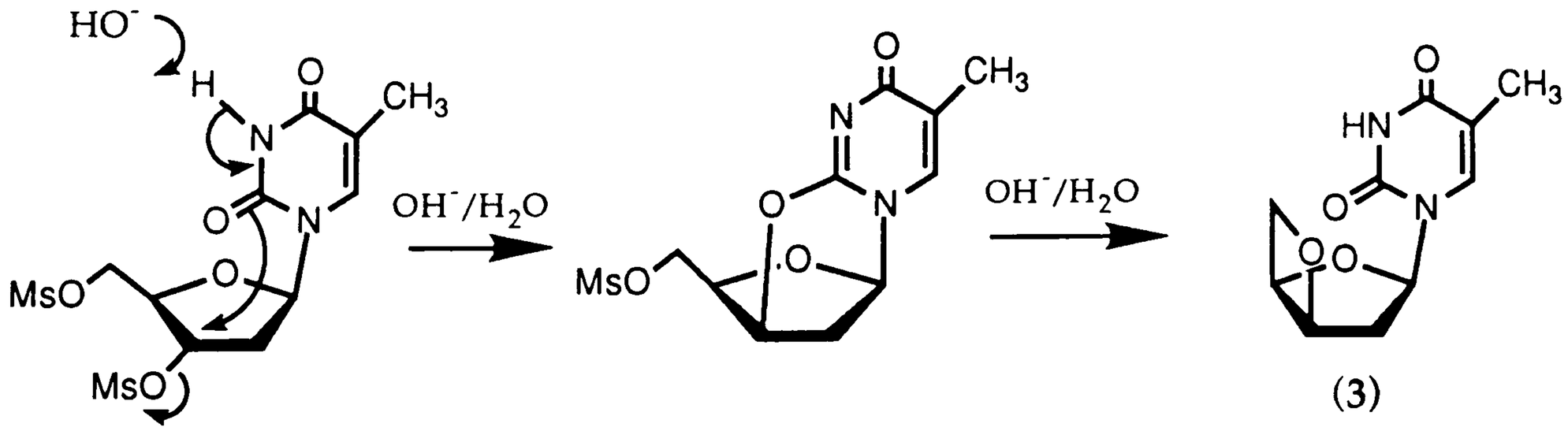
When thymidine (1) was heated with a four-fold excess of diphenyl sulphite at 156°C in dimethylacetamide solution, in the presence of a catalytic quantity of 1-methylimidazole, and the products were then subjected to a mildly alkaline hydrolytic work-up, 2,3′-anhydrothymidine (4) was obtained as a nearly pure white solid in 57% yield.



Scheme 2.4 Synthesis of 2,3′-anhydrothymidine (**4**)

The reaction is assumed to proceed via an intermediate cyclic sulphite. This converts the hydroxyl groups of the nucleoside into better leaving groups which are then displaced by intramolecular nucleophilic attack of O-2 on the β-face of the sugar. The 1-methylimidazole acts as a proton acceptor and catalyses the cyclisation step which yields the required 2,3′-anhydrothymidine (**4**).

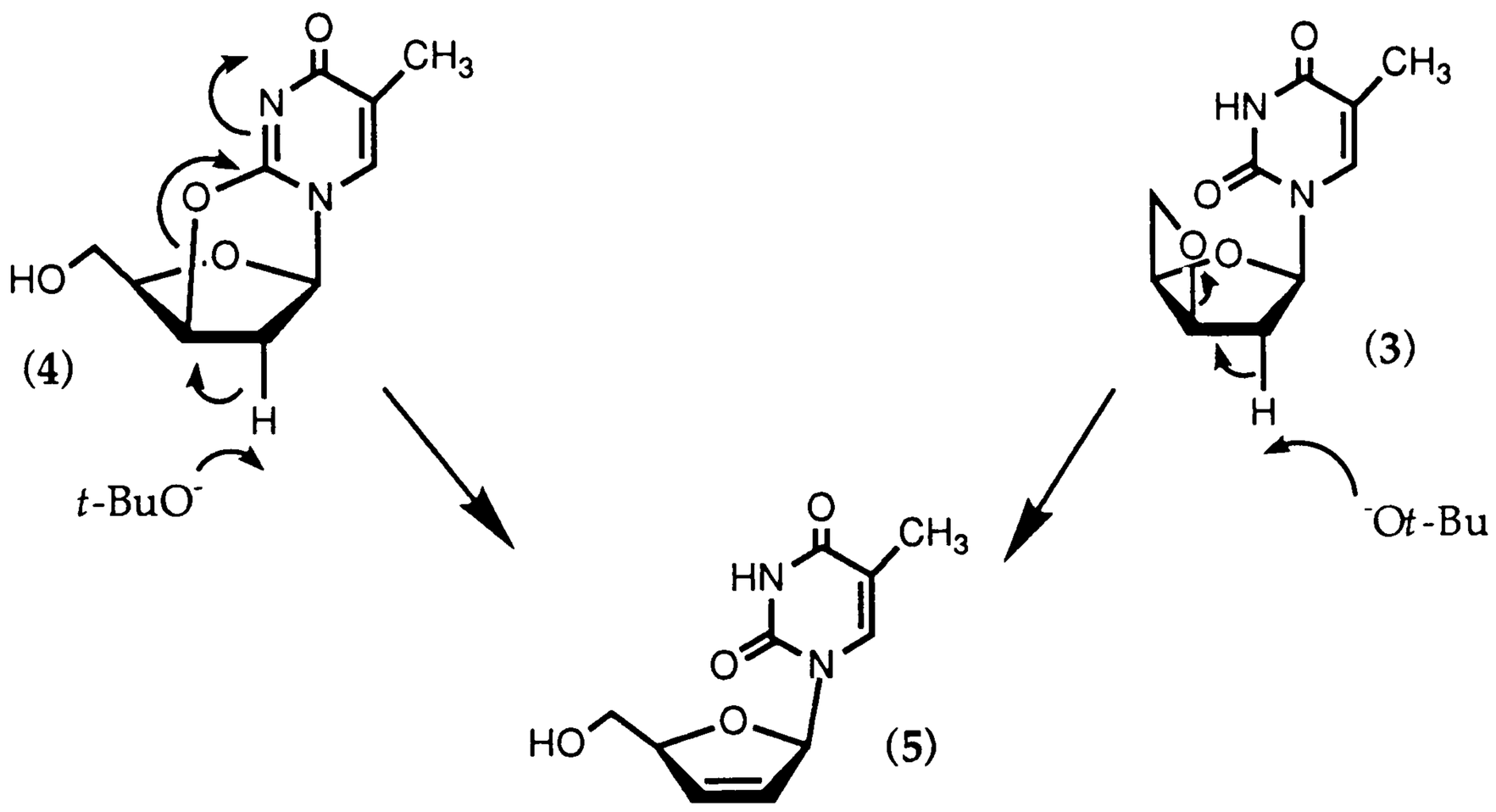
The reaction of thymidine with 2.5 equivalents of methanesulphonyl chloride gave the corresponding 3′,5′-di-O-mesyl derivative in nearly quantitative yield. This was converted to the 3′,5′-oxetane (**3**) derivative on treatment with aqueous sodium hydroxide.



Scheme 2.5 Synthesis of 3′,5′-cyclothymidine (**3**)

The reaction involves the intermediate formation of the 2,3′-anhydrothymidine, which on continued exposure to the basic reaction conditions results in the formation of the 3′,5′-oxetane (**3**) in 75% yield.

On treatment with potassium *t*-butoxide in dimethyl sulphoxide at ambient temperatures both the 2,3′-anhydrothymidine (**4**) and 3′,5′-oxetanothymidine (**3**) underwent ring opening of the ether moiety to yield the 2′,3′-unsaturated thymidine (**5**).

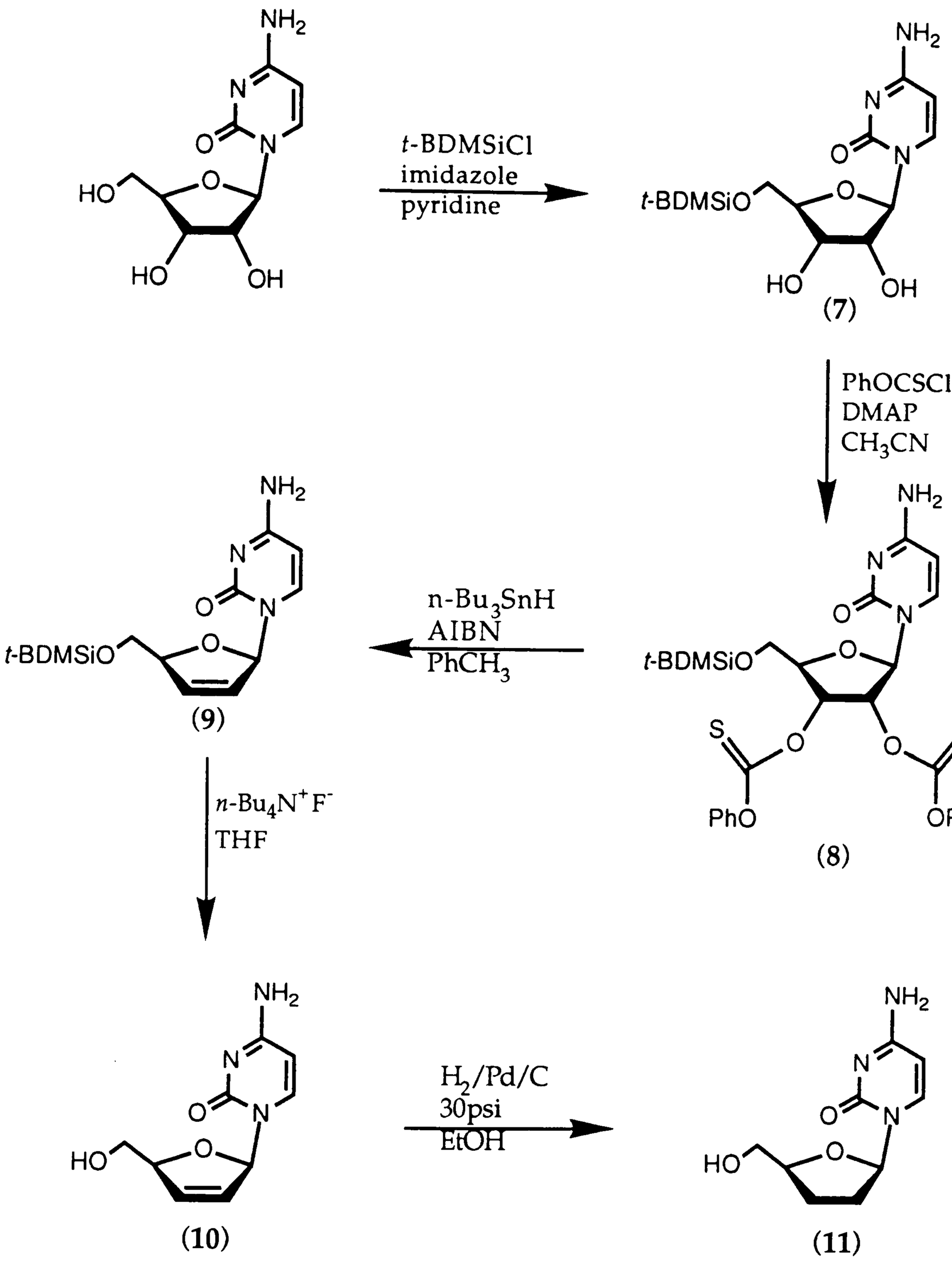


Scheme 2.6 Synthesis of 1-(2',3'-dideoxy-β-D-glycero-pento-2'-enofuranosyl)thymine (**5**)

Due to problems of removal of DMSO the yields of this step varied but it appeared that overall the two-step synthesis involving the 2,3′-anhydrothymidine intermediate gave the best yields. Catalytic hydrogenation as the final step yielded the required 3′-deoxythymidine (**6**) in 67% yield. The overall yields were 27%, via 2,3′-anhydrothymidine, and 21%, via 3',5′-cyclothymidine.

2.2.1.2: Synthesis of 2′,3′-dideoxycytidine (11)

To date, the most favoured method for the synthesis of 2′,3′-dideoxynucleosides is from 2′-deoxynucleosides by Barton-type deoxygenation reactions[144, 153] or from intact nucleosides by routes involving deoxygenation reactions to yield 2′,3′-unsaturated dideoxynucleosides, which are then hydrogenated. The conversion of cytidine to 2′,3′-dideoxycytidine by protection of the 5′-hydroxyl function, thioacylation, radical-induced reductive cleavage, deprotection and hydrogenation combines many of the literature methods[142, 143] to provide an efficient five-step synthesis.



Scheme 2.7 Total synthesis of 2′,3′-dideoxycytidine (**11**)

The initial protection of the 5′-hydroxyl group with *tert*-butyldimethylsilyl chloride proceeded in high yield. The imidazole acted as a proton acceptor and activated the 5′-hydroxyl group to nucleophilically attack the *tert*-butyldimethylsilyl chloride and displace the chloride ion.

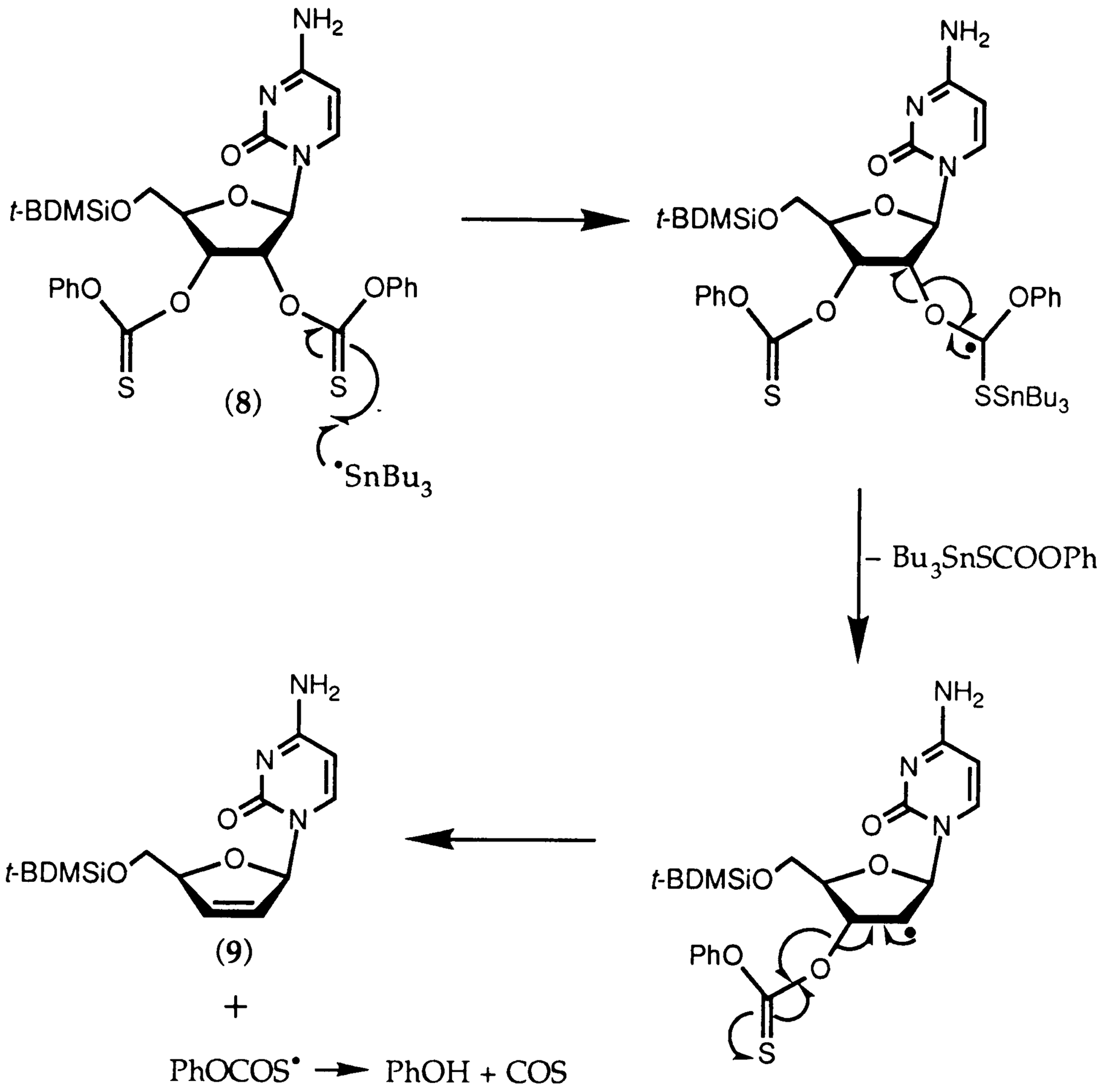
Free radical reduction of 2′- and 3′-hydroxy groups was used for several reasons[142]:

- the nucleophilic S_N2 displacement at the C-2′ functional group of nucleosides is inhibited by steric and electronic factors;
- generation of a cation, by an S_N1 reaction, at C-2′ is precluded by bonding to the adjacent electron-deficient anomeric carbon.
- generation of an anion at C-2′ would result in cleavage of the glycosidic bond.

Thus, homolytic cleavage of the C-2′–O-2′ bond is the only feasible method. The phenyl chlorothionocarbonate was the favoured reagent as it can be introduced into the nucleoside by a simple acylation reaction in good yield[142]. Pyridine can be used to catalyse the acylation of relatively unhindered alcohols, but for the rapid and high yielding conversion of nucleosides to their corresponding O-phenoxythiocarbonyl derivatives, 4-(dimethylamino)pyridine (DMAP) is required. This base is superior to pyridine as a catalyst for O-acylations of alcohols, particularly for tertiary and sterically hindered alcohols. The enhanced activity over pyridine is not a result of increased basicity (DMAP, pKa=9.70; Pyridine, pKa=5.29), but is due partly to the formation of high concentrations of N-acylpyridinium salts, which are very effective acylating reagents[154]. Thus, the N-thioacylpyridinium salt formed was open to nucleophilic attack by the 2′- and 3′-hydroxyl groups to give the thiocarbonate in 88% yield.

The reductive cleavage of the 5′-O-(*tert*-butyldimethylsilyl)-2′,3′-bis-O-(phenoxythiocarbonylcytidine) (**8**) occured readily in the presence of tri-*n*-butyltin hydride in toluene under reflux with addition of α,α′-

azobis(isobutyronitrile) as the radical initiator to give 2′,3′-didehydro-2′,3′-dideoxycytidine (**9**).



Scheme 2.8 Reductive cleavage of the thiocarbonate

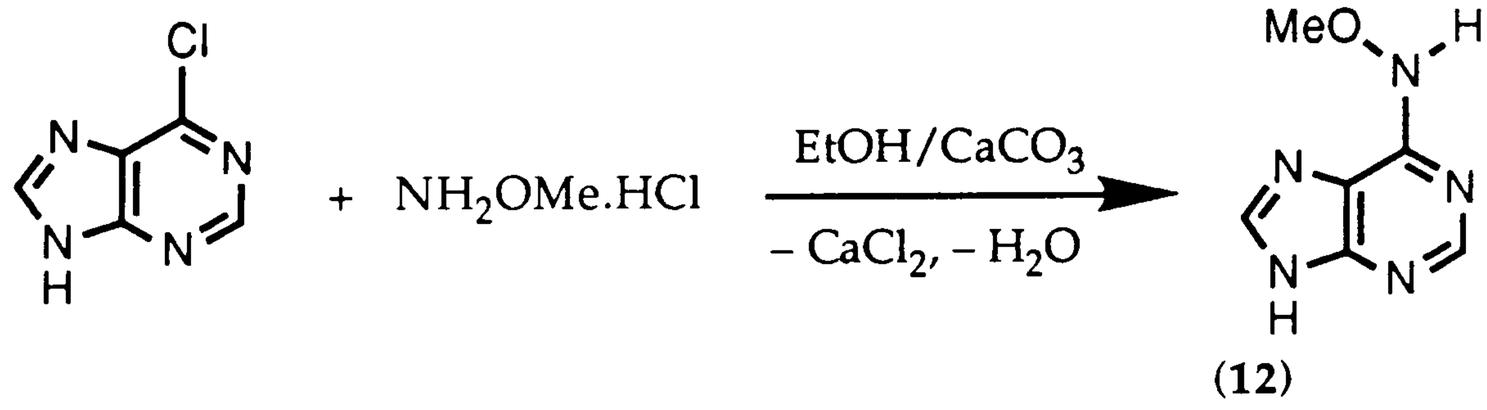
The vicinal diols were converted in high yield into an olefin by the reaction of tri-n-butyltin hydride with the bisdithiocarbonates. Previous studies have shown that the reaction proceeds by a stepwise radical fragmentation[144, 153]. Therefore, substitution in the β-position of the intermediate radical by a leaving group, which could leave as a stable radical, permitted the synthesis of the required alkene in 72% yield.

As catalytic hydrogenation is sensitive to steric hindrance, the unsaturated nucleoside was desilylated before the olefin was reduced. Deprotection was effected in nearly quantitative yield to give the 2′,3′-didehydro-2′,3′-dideoxycytidine (**10**) which was then reduced to afford an 88% yield. The overall yield of 2′,3′-dideoxycytidine (**11**) was 41% compared to the 27% overall yield of 3′-deoxythymidine.

Synthesis of Base Acceptors

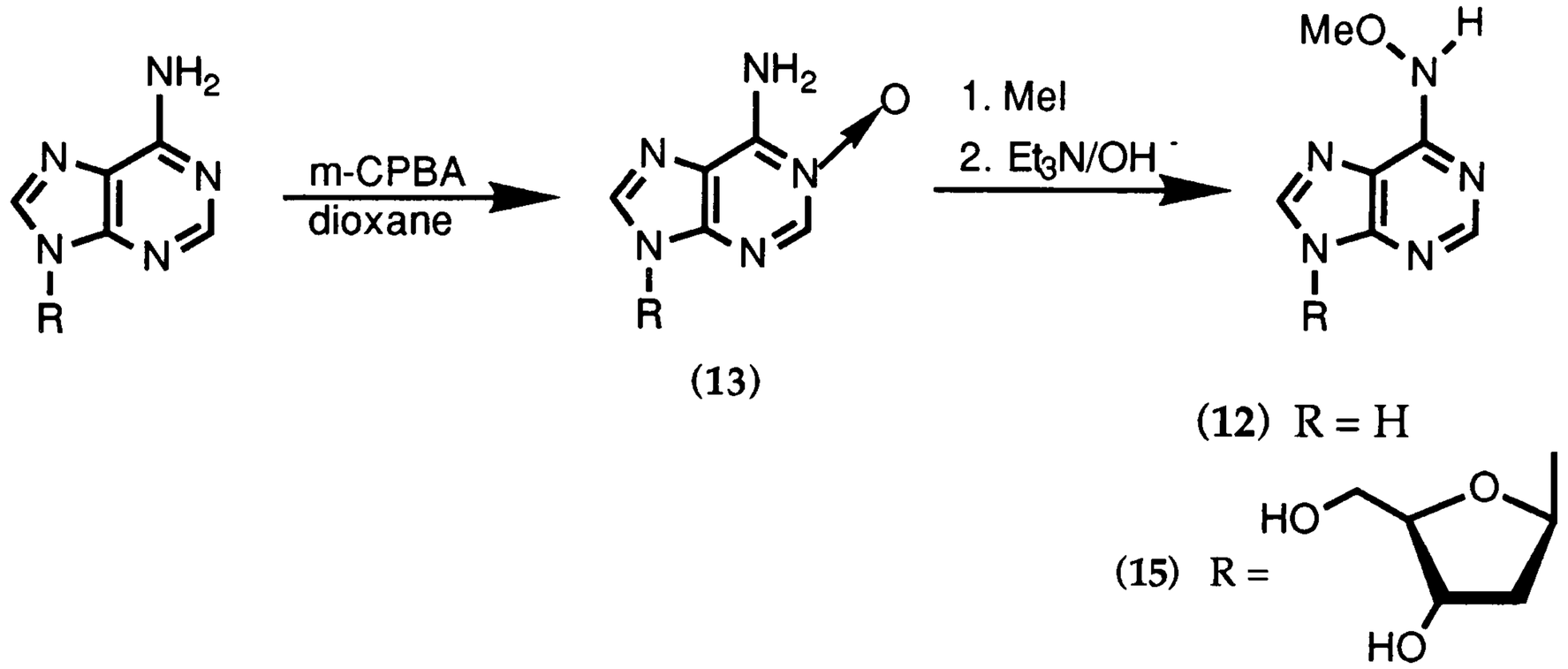
2.2.1.3: Synthesis of N^6-methoxyadenine (12)

O-Methylhydroxylamine and hydroxylamine are highly efficient mutagens in many biological systems[155, 156]. Therefore, it was of interest to synthesise the N^6-methoxyadenine 2′,3′-dideoxynucleoside to investigate its anti-HIV properties. Initial attempts to synthesise N^6-methoxyadenine (**12**) by the method of Budowsky *et al.*[155], where the O-methylhydroxylamine directly substitutes the amino group in the adenine, proved to be low yielding. The method of Robins[157], was applied to our system where 6-chloropurine was heated with methoxyamine hydrochloride in ethanol to displace nucleophilically the chlorine atom and yield N^6-methoxyadenine (**12**) in 62% yield.



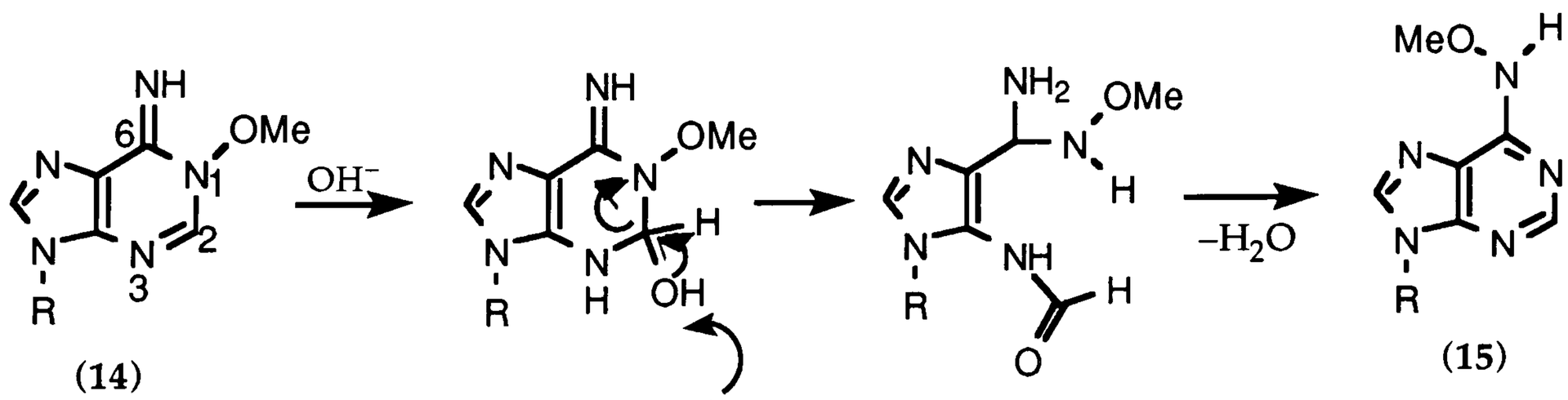
Scheme 2.9 Methoxyamination of 6-chloropurine

The method of Saneyoshi *et al.*[158] was applied to both adenine and 2′-deoxyadenosine. Reaction of the 1-oxide (**13**) with methyl iodide followed by alkaline treatment resulted in a rearrangement to give the required N^6-methoxy derivative (**15**).



Scheme 2.10 Methoxyamination of adenine and 2′-deoxyadenosine

The initial oxidation step with *m*-chloroperbenzoic acid in aqueous dioxane gave the 1-oxide in high yield. This was then reacted with methyl iodide in dimethylformamide to give the 1-methoxy derivative (**14**) which was not isolated. Heating at 60°C under basic conditions facilitated a Dimroth rearrangement to yield the N^6-methoxy derivative (**15**).



Scheme 2.11 Dimroth rearrangement of 1-methoxyadenine (**14**) to N_6-methoxyadenine (**15**)

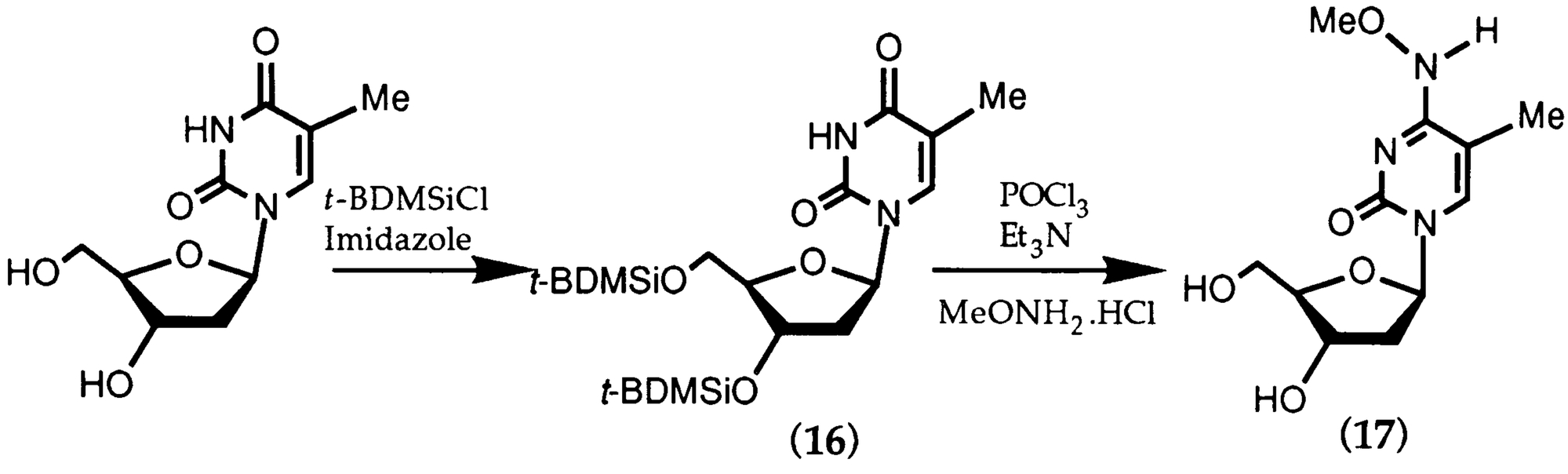
In the Dimroth rearrangement the first step is the rapid addition of the base across the most electrophilic C=N bond (in this case, the 2- and 3-positions), resulting in a ring carbon atom having three electron-withdrawing substituents. This situation leads to hydrolytic ring fission (at the N1,C2-bond) to expose an amidine group. The final step is the slow ring closure, after rotation of the amidine group, and the expulsion of the facilitating base.

Initially this method was used on 2′-deoxyadenosine and then the glycosyl bond was hydrolysed, by the use of solid phase acid in the form of Dowex H^+, to liberate the free N^6-methoxyadenine from the 2-deoxyribose. It was subsequently found that the method could be applied to adenine to give the free N^6-methoxyadenine straight away, for use in the enzyme transfer reactions.

2.2.1.4: Synthesis of N^4-methoxy-5-methylcytidine (17)

For the same reasons, N^4-methoxy-5-methylcytosine (**18**) was synthesised also for use in the enzymatic synthesis of the N^4-methoxy-5-methylcytosine 2′,3′-dideoxynucleoside, to be investigated for possible anti-HIV properties.

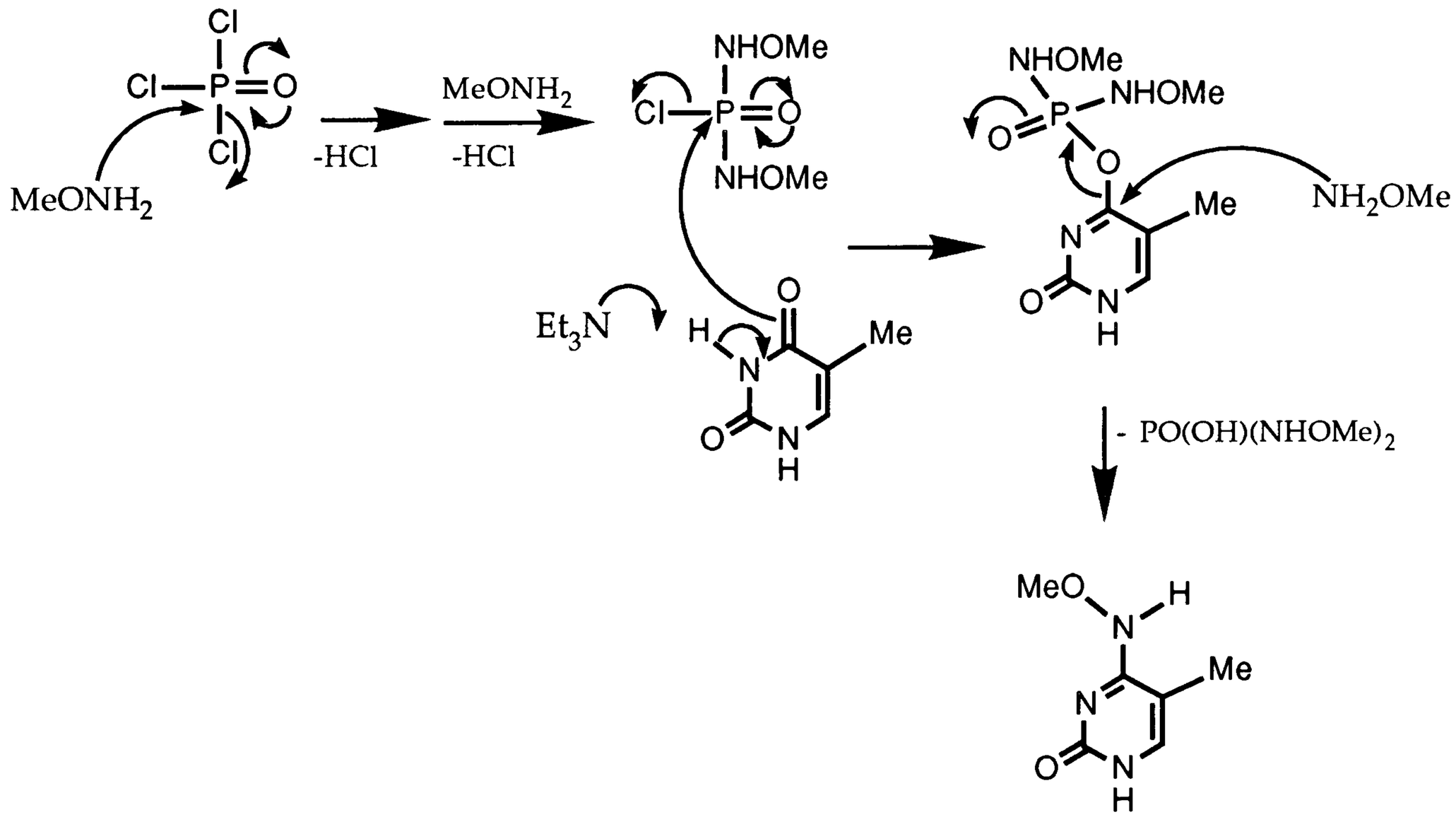
The method of Xu[159] was adapted for the synthesis of N^4-methoxy-5-methylcytidine (**17**) in 81% overall yield. The 3′- and 5′-hydroxyl groups of thymidine were first protected with the *tert*-butyldimethylsilyl groups in high yield, before the substitution of the C-4 carbonyl group for the methoxyamino group. The silyl protecting groups were removed during the work-up and purification steps therefore, there was no need to use tetra-*n*-butylammonium fluoride in a deprotection step.



(16) (17)

Scheme 2.12 Synthesis of N^4-methoxy-5-methylcytidine (17)

The same method was also used on thymine to yield the required N^4-methoxy-5-methylcytosine (**18**), thus avoiding the need to hydrolyse the thymidine derivative to liberate the free base.



(18)

Scheme 2.13 Synthesis of N^4-methoxy-5-methylcytosine (18)

The substitution step involved the initial formation of the phosphorus intermediate by nucleophilic attack of the excess methoxylamine on the phosphorus oxychloride. After 0.5 hours, the thymine was added to react with the phosphorus intermediate and thus transform the C-4 carbonyl

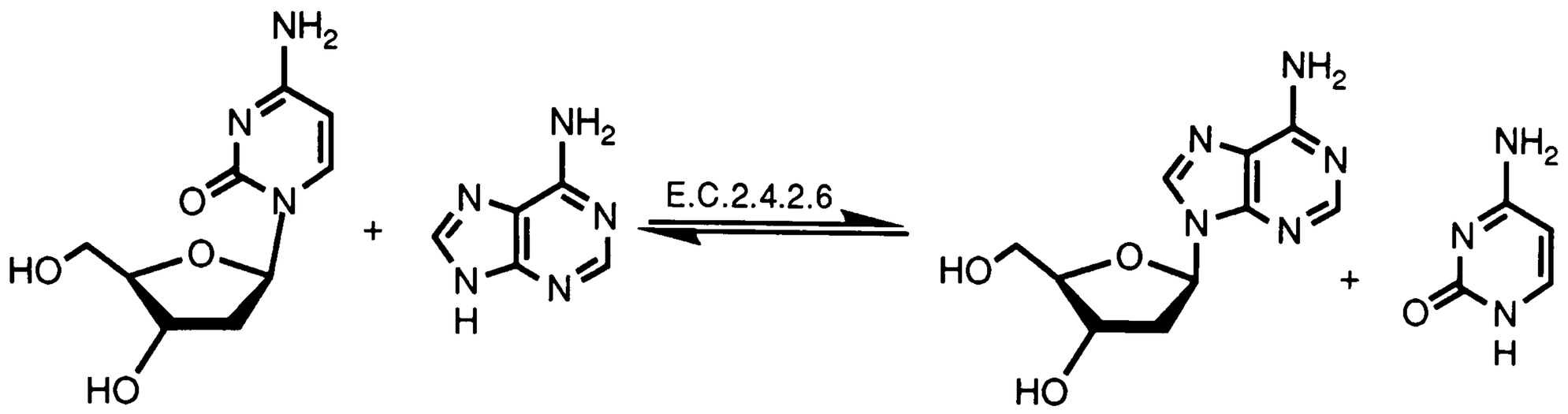
group into a good leaving group, for the subsequent nucleophilic attack of the methoxylamine base to give the product in 72% yield.

2.2.2: Enzymatic Synthesis of Nucleoside Analogues

2.2.2.1: Purification of nucleoside N-deoxyribosyltransferases (E.C.2.4.2.6) from *Lactobacillus leichmannii*

Bacteria are one of the most favoured sources of enzymes and the purification of a crude preparation of N-deoxyribosyltransferases from lactic acid bacteria was fairly simple. *Lactobacillus leichmannii* was grown for 24 hours at 37°C. The cells were then harvested by centrifugation during their stationary phase, when the highest levels of N-deoxyribosyltransferases are present. The cells were broken open by the use of a French pressure cell, which forces the cell suspension through a restricted orifice at high pressure. After cell breakage, the cell debris was removed by centrifugation. The resulting yellow liquid was dialysed to remove salts and thus yielded the crude preparation of enzyme. However, this preparation contained a multitude of different enzymes which could interfere with the transfer reactions. To remove the need for any further purification steps a technique, whereby the other enzymes in the crude preparation are inhibited by the addition of 10% ethylene glycol, has been developed[126]. This crude enzyme preparation was then assayed for its protein concentration using the dye-binding BioRad protein assay, and assayed to determine the number of units present.

The basic enzymatic transfer reaction involves the exchange of the base moiety in the nucleoside with a free base molecule to yield a new nucleoside.



dC A dA C

Scheme 2.14 Standard enzymatic transfer reaction

The standard assay system used to calculate the number of units present is as shown above: 2′-deoxycytidine (dC) is the glycosyl donor while adenine (A) acts as the glycosyl acceptor. The rate at which the new nucleoside, 2′-deoxyadenosine (dA), was produced was followed by reverse-phase HPLC, detected by UV at 254nm. It is important to remember that the units and specific activity of a sample of enzyme will vary with the assay system used. Therefore, a unit of enzyme must be defined for the particular assay system used, as can be found in the Experimental section. An example of the different substrate specificities and rates of reaction is clearly seen when several nucleosides and bases are investigated as donors and acceptors for the transfer reaction[130].

2.2.2.2: Comparison of glycosyl donors

The transfer reaction is an equilibrium and therefore, if it is to have any use as a synthetic tool it must be displaced as fully as possible in the direction of the products. The adaptations which are generally used to push the equilibrium to the right-hand-side are:

- a ratio of 3:1 donor nucleoside to acceptor base;
- a donor nucleoside which has a high affinity for the enzyme;
- a donor nucleoside whose base is a good leaving group.

For the synthesis of 2′-deoxy- and 2′,3′-dideoxynucleosides, a glycosyl donor is required which will give high levels of transfer with a large number of acceptor bases. From substrate specificity studies performed it has been shown that 2′-deoxycytidine is a better 2-deoxyribosyl donor than thymidine, 2′-deoxyuridine or any of the other naturally occuring nucleosides[105]. A brief investigation and comparison of the glycosyl donor abilities of 2′-deoxycytidine, thymidine, 2′,3′-dideoxycytidine (**11**), and 3′-deoxythymidine (**6**) confirmed the findings for 2-deoxyribosyl donor:

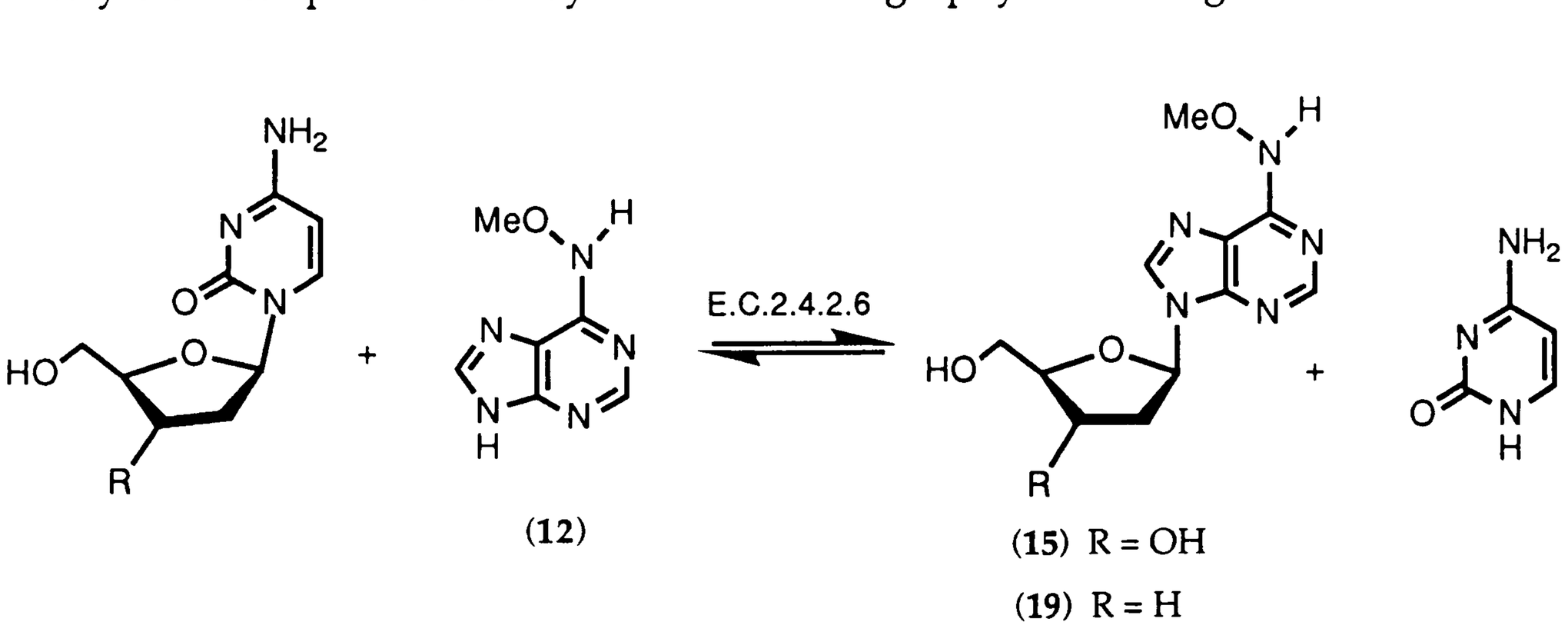
- the transfer with 2′-deoxycytidine and adenine was five times faster than the corresponding reaction with thymidine as the glycosyl donor and adenine as the glycosyl acceptor;
- the transfer with 2′-deoxycytidine and 2-aminopurine was four times faster than the corresponding reaction with thymidine as the glycosyl donor and 2-aminopurine as the glycosyl acceptor.

It was also discovered that the 2′,3′-dideoxynucleosides follow the same pattern, in that 2′,3′-dideoxycytidine (**11**) is a better 2,3-dideoxyribosyl donor than 3′-deoxythymidine (**6**). A study by Carson and Wasson[130] indicated that 2′,3′-dideoxycytidine and 3′-deoxythymidine were approximately equivalent as glycosyl donors, and were superior to the purine 2′,3′-dideoxynucleosides. However, 2′,3′-dideoxycytidine (**11**) was the best glycosyl donor when adenine was the acceptor base, as in our studies. Therefore, for the transfer reactions employing N-

deoxyribosytransferases, normally 2′-deoxycytidine and 2′,3′-dideoxycytidine were used as the glycosyl donors.

2.2.2.3: Synthesis of N^6-methoxy-2′-deoxyadenosine (15) and N^6-methoxy-2′,3′-dideoxyadenosine (19)

As discussed previously, these compounds are of interest as possible anti-HIV drugs because of the mutagenic properties of methoxylamine. Using a crude preparation of N-deoxyribosyltransferases from *Lactobacillus leichmannii* the transfer of 2-deoxyribose from 2′-deoxycytidine to N^6-methoxyadenine (**12**) in the presence of ethylene glycol occured in good yield. The ethylene glycol inhibits the degradative enzymes present in the crude N-deoxyribosyltransferase preparation. Only one compound was detected by HPLC. N^6-Methoxy-2′-deoxyadenosine (**15**) was synthesised in 72% yield after purification by flash chromatography on silica gel.



(12)

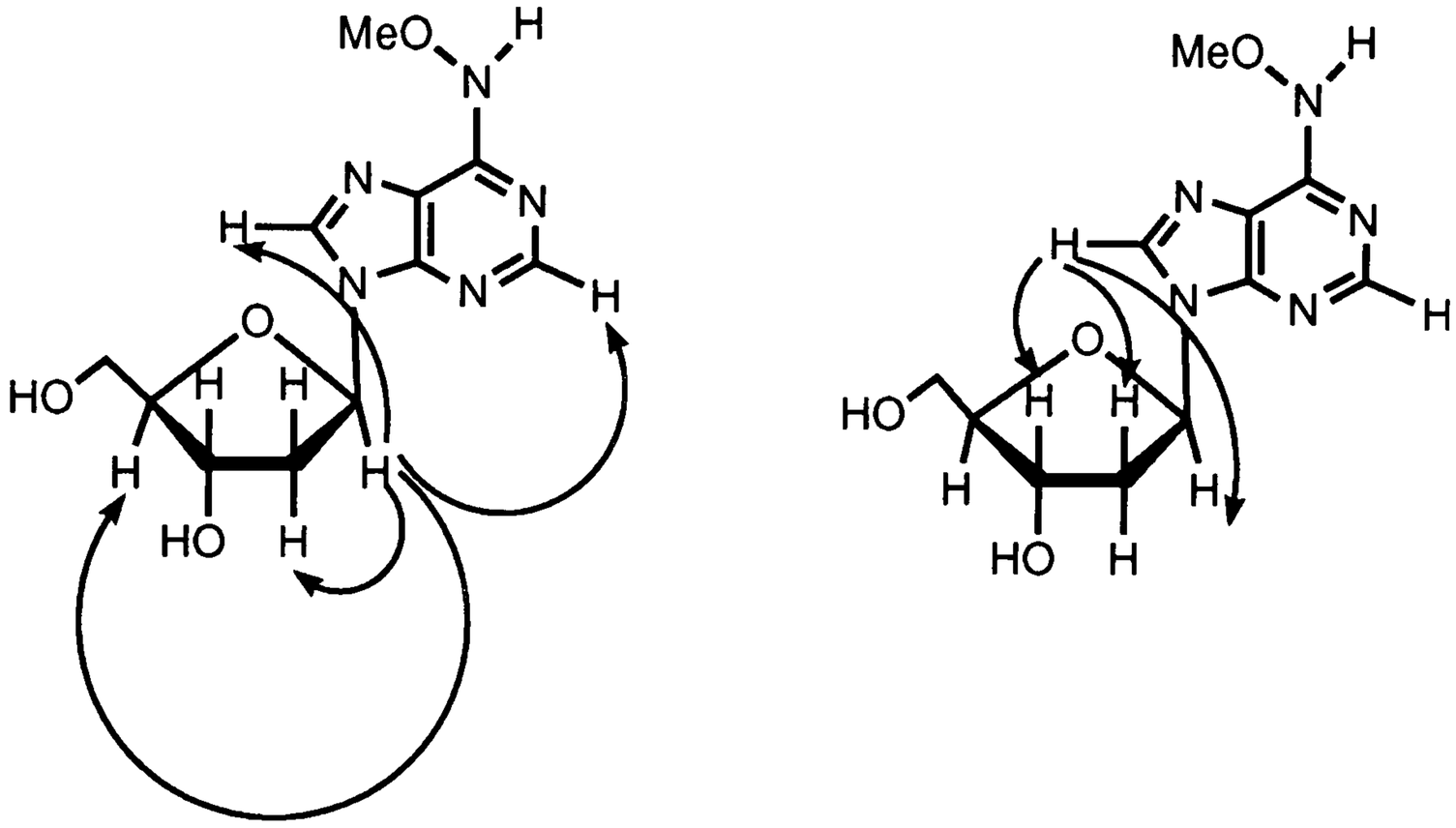
(**15**) R = OH

(**19**) R = H

Scheme 2.15 Enzymatic synthesis of N^6-methoxyamino-2′-deoxy- (**15**) and -2′,3′-dideoxyadenosine (**19**)

The structure of the N^6-methoxy-2′-deoxyadenosine (**15**) was confirmed by the use of nuclear Overhauser enhancement experiments, whereby a

signals in the spectrum are observed. This method confirmed that the new nucleoside not only had a β-glycosyl linkage of the sugar moiety to the heterocyclic base but also that the glycosyl bond had been formed to the correct nitrogen in the heterocyclic base, which in this instance was the N–9.



Irradiation at $H_{1'}$ Irradiation at H_8

Fig. 2.16 Sites of nuclear Overhauser enhancements in the ^1H NMR spectrum of N^6-methoxy-2′-deoxyadenosine (**15**)

Saturation of the signal due to $H_{1'}$ caused enhancement of the signal due to H_8, on the heterocyclic base as well as the signals due to $H_{4'}$ and $H_{2'b}$ on the underside of the 2-deoxyribose ring. No enhancements were observed for signals on the topside of the 2-deoxyribose ring. When the signal due to H_8 was saturated, the signals due to $H_{1'}$ as well as those due to $H_{3'a}$ and $H_{2'a}$ on the topside of the 2-deoxyribose ring were enhanced. This confirmed that the nucleoside has a β-glycosyl bond and indicated that the N^6-methoxyadenine lay at an angle to the sugar moiety.

Irradiation at $H_{1'}$ (6.29ppm)

	% Enhancement at		
Compound	H-8	H-2'b(down)	H-4'
15	4.9	6.1	1.8

Irradiation at H_8 (8.15ppm)

	% Enhancement at		
Compound	H-1'	H-2'a(up)	H-3'a(up)
15	3.8	1.2	1.1

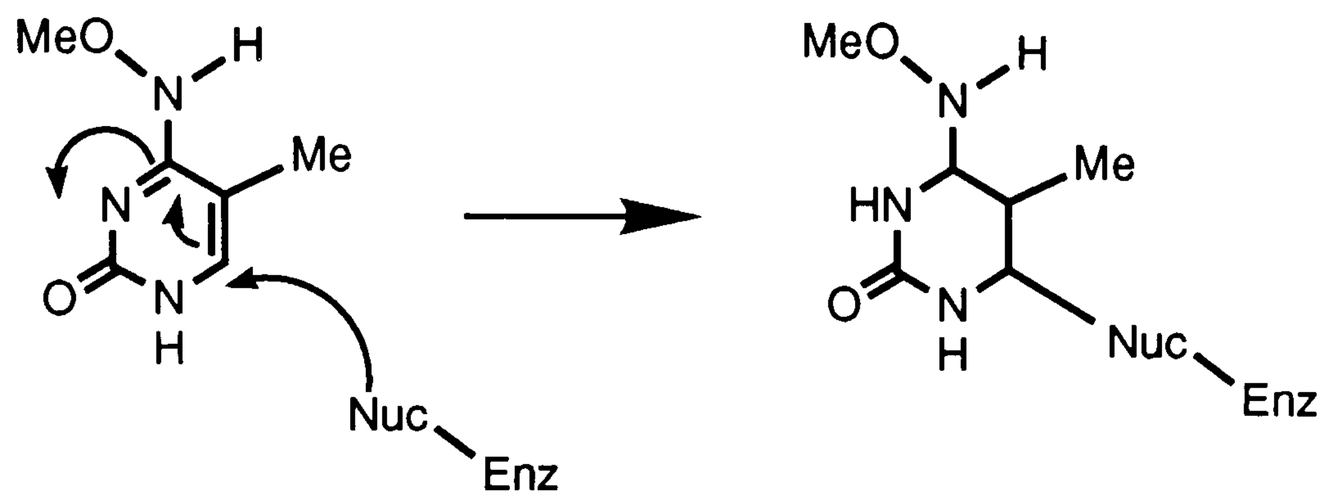
Table 2.1 Nuclear Overhauser enhancements (%) caused by irradiating signals in the ^1H NMR spectrum of N^6-methoxy-2'-deoxyadenosine (**15**)

Confirmation of the position of attachment of the 2-deoxyribosyl residue at the N-9 in a β-configuration was also made by comparison of the ^1H NMR spectrum of this compound with the ^1H NMR spectrum of the chemically synthesised nucleoside.

However, the equilibrium of the transfer of the 2,3-dideoxyribosyl moiety to N^6-methoxyadenine was unfavourable and there was no significant amount of the 2',3'-dideoxynucleoside formed.

2.2.2.4: Synthesis of N^4-methoxy-5-methylcytidine (17)

The N^4-methoxy-5-methylcytosine (**18**) was synthesised to compare with N^6-methoxyadenine as a 2-deoxy- and 2,3-dideoxyribose acceptor and to investigate any anti–HIV activity. Unfortunately no transfer was observed for either reaction.



Scheme 2.17 Possible reaction of N^4-methoxy-5-methylcytosine (**18**) at the active site of an enzyme

The possible inhibitory effects of N^4-methoxy-5-methylcytosine (**18**) were investigated by incubation of the compound with the crude N-deoxyribosyltransferase and then assaying the enzyme for activity after certain intervals of time. No loss in activity was observed so inhibition did not appear to be a factor for the lack of transfer.

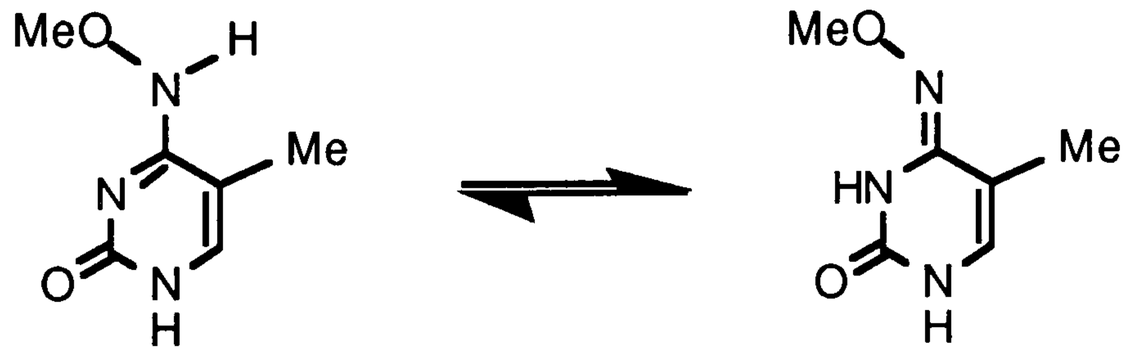
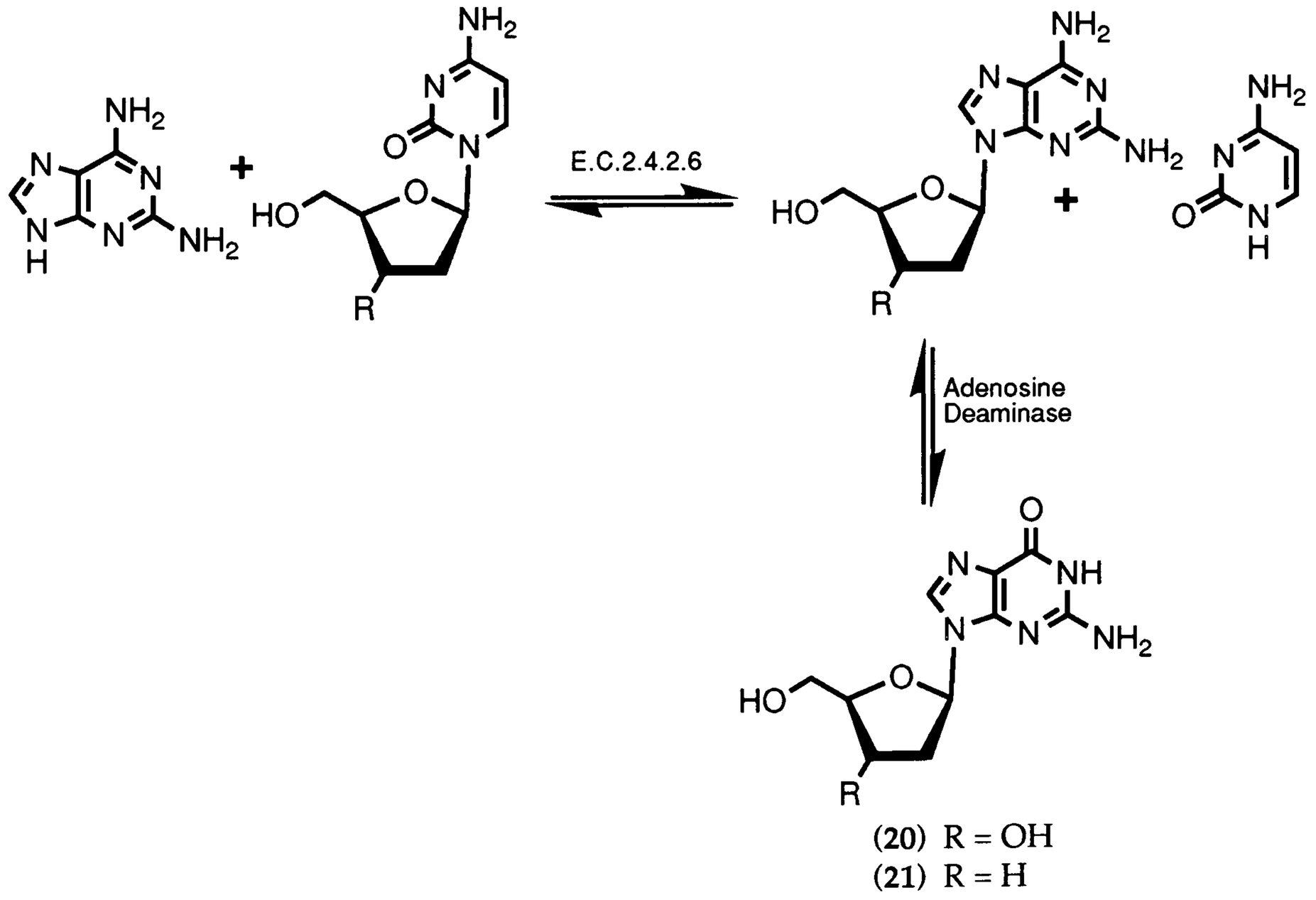


Fig. 2.18 Structure of N^4-methoxy-5-methylcytosine (**18**)

The reason for the lack of transfer in the reaction for the formation of N^4-methoxy-5-methylcytidine is thus postulated to be due to the low nucleophilicity of the acceptor base in the reaction. The pyrimidine base now has two electron withdrawing groups, the C–2 carbonyl and the C–4 methoxyamine, which reduce the nucleophilicity of N–1 dramatically and render it as a very poor substrate.

2.2.2.5: Synthesis of 2′-deoxy- (20) and 2′,3′-dideoxyguanosine (21)

Guanine, guanosine and their derivatives are very insoluble in aqueous and alcoholic solution which can cause many problems in any synthesis involving these compounds. Therefore, it was felt that a procedure involving a masked guanosine derivative with good solubility properties, which could be converted to the guanosine derivative as the final step, would provided an excellent pathway to synthesise a whole selection of different derivatives in good yields. There have been several chemical methods published which involve the initial chemical synthesis of a masked nucleoside derivative which is then treated with adenosine deaminase as the final step to yield the required nucleoside derivative[87, 160].



Scheme 2.19 Synthesis of guanosine derivatives (**20**) and (**21**) by a coupled-enzyme system

A crude preparation of N-deoxyribosyltransferases from *Lactobacillus leichmannii* was employed in the transfer of 2-deoxyribose from 2′-deoxycytidine to 2,6-diaminopurine in the presence of ethylene glycol. When the reaction had reached ~100% transfer in the production of the 2,6-diaminopurine 2′-deoxynucleoside, the pH was adjusted to 7.4 and adenosine deaminase was added. This yielded the required 2′-deoxyguanosine (**20**) in 93% yield, after purification by ion exchange chromatography. Only one final product was detected by HPLC.

The structure of the 2′-deoxyguanosine (**20**) was confirmed by the use of nuclear Overhauser enhancement experiments as described previously. The ^{1}H NMR spectrum was also compared to the spectrum of an authentic sample.

Irradiation at $H_{1'}$ (6.22ppm)

	% Enhancement at		
Compound	H-8	H-2′b(down)	H-4′
20	2.5	5.1	1.1

Irradiation at H_8 (7.83ppm)

	% Enhancement at		
Compound	H-1′	H-2′a(up)	H-3′a(up)
20	4.9	3.9	2.8

Table 2.2 Nuclear Overhauser enhancements (%) caused by irradiating signals in the ^{1}H NMR spectrum of 2′-deoxyguanosine (**20**)

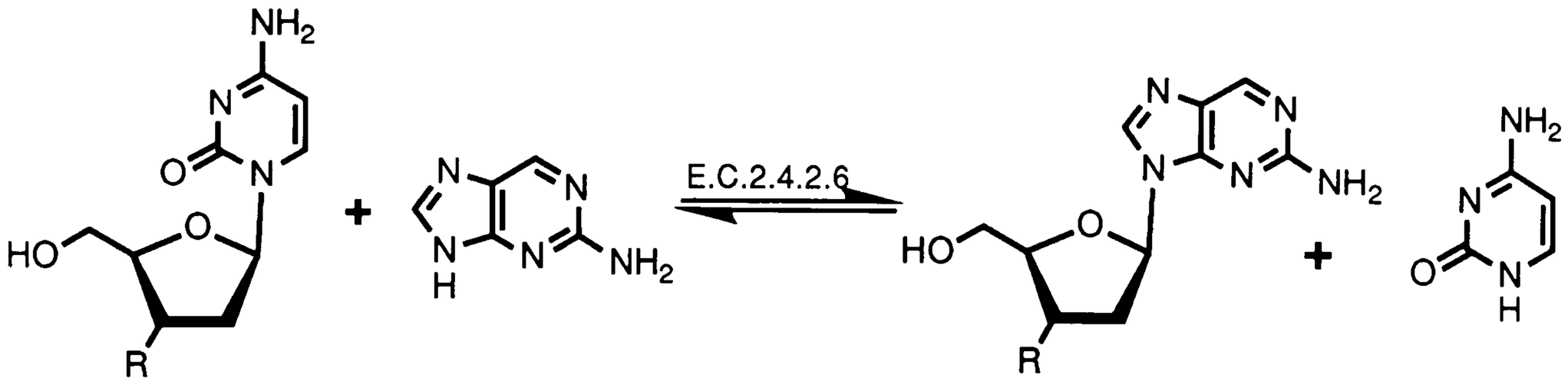
Unfortunately, the transfer of 2,3-dideoxyribose from 2′,3′-dideoxycytidine to 2,6-diaminopurine using a crude preparation of N-deoxyribosyltransferase from *Lactobacillus leichmannii* was unsuccessful.

It was therefore felt that further investigations into this procedure with the transfer reaction as the first step were not worthwhile.

It is interesting to note the many conflicting results presented to date regarding the ability of 2,6-diaminopurine to act as a glycosyl acceptor in these deoxyribose transfer reactions. 2,6-Diaminopurine has been reported to give negative results with extracts of *Lactobacillus helveticus*[99], *Thermobacter acidophilus*[104], and *L. leichmannii*[161]. However, it has been also be found to act as a competent substrate with extracts of *L. delbruecki*[161], *L. helveticus*[113] and *L. leichmannii*[114].

2.2.2.6: Synthesis of 9-β-D-2′-deoxy- (22), and 9-β-D-2′,3′-dideoxyribofuranosyl 2-aminopurine (23)

A crude preparation of N-deoxyribosyltransferase from *Lactobacillus leichmannii* was used to transfer the 2-deoxyribosyl and 2,3-dideoxyribosyl residues from 2′-deoxycytidine and 2′,3′-dideoxycytidine, respectively, to 2-aminopurine. Ethylene glycol was added to the reaction mixture to inhibit hydrolase and deaminase activities in the crude enzyme preparation. After purification by flash chromatography on silica gel the 2′-deoxy- (**22**) and 2′,3′-dideoxyribonucleosides (**23**) of 2-aminopurine were prepared in 57% and 80% yield respectively.



(22) R = OH
(23) R = H

Scheme 2.20 Synthesis of the 2′-deoxy- (**22**) and 2′,3′-dideoxyribonucleosides (**23**) of 2-aminopurine

2-Aminopurine contains five nitrogen atoms and a mixture of products may be expected to result from these experiments. Only one product was formed in each of the synthetic reactions. The structures of these nucleosides were confirmed by high field NMR spectroscopy, in particular by using nuclear Overhauser enhancement experiments to demonstrate the β-configuration at the glycosidic centre and the attachment of the glycosyl residue to N-9. Thus, irradiation of the signal due to $H_{1'}$ in the ^1H NMR spectra of nucleosides (**22**) and (**23**) enhanced *inter alia* the signals due to $H_{2'b}$ and $H_{4'}$. Therefore, $H_{1'}$, $H_{2'b}$ and $H_{4'}$ must be on the underside of the glycosyl ring which confirms the presence of the β-glycosidic link. Irradiation of the signal due to H_8 enhanced the signals due to $H_{2'a}$ and $H_{3'a}$ which are on the topside of the glycosyl ring. The signal due to $H_{1'}$ was also enhanced and the magnitude of the enhancements observed suggests a *syn*-conformation for the nucleosides. Irradiation of the signal due to H_6 in both nucleosides caused no enhancement of any of the signals due to sugar protons. This confirmed that the position of attachment of the glycosyl rings was at N-9, as attachment at N-7 might be expected to give rise to nOe enhancements of signals due to sugar protons when the signal due to H_6 was irradiated.

Irradiation at $H_{1'}$

	% Enhancement at		
Compound	H-8	H-2'b(down)	H-4'
22	3.6	6.1	0.7
23	1.2	6.1	0.9

Irradiation at H_8

	% Enhancement at		
Compound	H-1'	H-2'a(up)	H-3'a(up)
22	4.7	1.7	0.6
23	2.1	0.8	0.4

Table 2.3 Nuclear Overhauser enhancements (%) caused by irradiating signals in the ^1H NMR spectrum of the 2'-deoxy- (**22**) and 2',3'-dideoxyribonucleosides (**23**) of 2-aminopurine

9-β-D-2',3'-Dideoxyribofuranosyl-2-aminopurine (**23**) was assayed to investigate any anti-HIV or anti-SIV activities or toxicities[162]. It was found to selectively inhibit the replication of HIV–1, HIV–2, and SIV which were measured by examining syncytia formation, total cell viability and gp120 production. The EC_{50} values, based on reduction in gp120 synthesis, were in the region of 8–40μM depending on the virus strain and cell type. The antiviral activity of (**23**) was shown to be similar, though somewhat less than that of ddA particularly in JM cells where its activity was reduced like that of AZT. 9-β-D-2',3'-Dideoxyribofuranosyl-2-aminopurine (**23**) lacked the toxicity of ddC as its TC_{50} values were >1000μM in both cell lines.

Virus	Compound	C8166 Cells /μM		JM Cells /μM	
		EC_{50}	TC_{50}	EC_{50}	TC_{50}
$HIV\text{-}1_{IIIB}$	**23**	40	>1000	8	>1000
	ddA	4	>1000	1.6	>1000
	ddC	0.05	>1000	0.05	0.5
	AZT	0.016	>1000	100	>1000
$HIV\text{-}2_{ROD}$	**23**	100	>1000		
	ddA	4	>1000		
	ddC	0.05	>1000		
	AZT	0.016	>1000		
SIV_{Mac}	**23**	80	>1000		
	ddA	4	>1000		
	ddC	0.05	>1000		
	AZT	0.016	>1000		

EC_{50} represents the concentration of compound that decreases gp120 antigen production in infected cells to 50% of control. The values in the table are the mean of three determinations. The cytotoxicity (TC_{50}) values for compound (**23**) were >1000μM, we were unable to test at higher concentrations than 1000μM.

Table 2.4 Median effective (EC_{50}) doses of 9-β-D-2′,3′-dideoxyribofuranosyl-2-aminopurine (**23**) for inhibition of HIV and SIV

This investigation indicated that 9-β-D-2′,3′-dideoxyribofuranosyl-2-aminopurine (**23**) had appreciable anti–HIV-1 activity together with some anti–HIV-2 and anti–SIV activity even though it lacks an oxygen, nitrogen, or chlorine substituent at the 6-position in the purine ring and appeared from spectroscopic studies to have the *syn*-conformation. There has been little published on the effect of *syn*- and *anti*-conformations of bases in anti–HIV nucleosides. However, the influence on the anti–HIV activity of the shapes of the sugar rings of such nucleosides, as determined in the solid state by X–ray crystallography, has been discussed[163]. These results suggest that an *anti*-conformation for the nucleoside in solution is

not essential. While crystallographic data gives information about nucleosides in solid state, it is important to remember that in solution the nucleoside does not possess a unique rigid structure but is in a dynamic equilibrium between many rapidly interconverting conformations. More information and discussion about nucleoside conformation will be presented in Chapter 4.

The published data on the anti–HIV activity of the only other 2′,3′-dideoxynucleoside examined which lacks a substituent in the 6-position of the purine ring system, 9-β-D-2′,3′-dideoxyribofuranosyl purine, is conflicting. One report suggests that it is active as an inhibitor of HIV replication[148], while another report indicates that it is inactive (Chu *et al.*, personal communication). This discrepancy probably lies in the difference in cell lines used to study the activities of the compounds, as the assay for 9-β-D-2′,3′-dideoxyribofuranosyl-2-aminopurine (**23**) indicated a noticeable difference in activity of the compound in the two cell lines which were used.

2.2.2.7: Synthesis of 2-Thio-2′-deoxyuridine (24)

2-Thio-2'-deoxyuridine (**24**) was required for synthesising base modified sections of DNA, so a large scale synthesis was required. Therefore, the synthesis was investigated on a small scale using a soluble extract of N-deoxyribosyltransferase from *Lactobacillus leichmannii* prior to a large scale synthesis using a poly(acrylamide-*co*-N-acryloxysuccinimide) (PAN) immobilised extract of the enzyme (as described in Chapter 3). After purification by flash chromatography on silica gel to give 2-thio-2′-deoxyuridine in 47% yield, the structure was assigned using NMR and

nuclear Overhauser enhancement experiments by the methods discussed previously.

2.2.3: Conclusion

The successful synthesis of 2′,3′-dideoxycytidine (**11**) and 3′-deoxythymidine (**6**) as glycosyl donors and the N^6-methoxyadenine (**12**) and N^4-methoxy-5-methylcytosine (**18**) as glycosyl acceptors was demonstrated. From these studies it can be seen that the enzyme does have a high degree of selectivity for both modified sugars and bases. The transfer of the 2,3-dideoxyribosyl moiety is always much slower than the corresponding transfer of the 2-deoxyribosyl moiety and if the acceptor base contains any unusual features this can often create an unfavourable equilibrium and slow down or even stop the transfer reaction. The synthesis of N^4-methoxy-5-methylcytidine (**17**) was unsuccessful due to the low nucleophilicity of the acceptor base, N^4-methoxy-5-methylcytosine (**18**), and not because of irreversible inhibition at the active site of the enzyme.

The problem of unfavourable equilibrium was encountered in the synthesis of 2′,3′-dideoxyguanosine (**21**). As a result of the difficulty in synthesising nucleoside analogues with 2– and 6–substituted purine base an investigation into the transfer rates of 2,6-disubstitued purines was undertaken and the results are tabulated in Appendix 1.

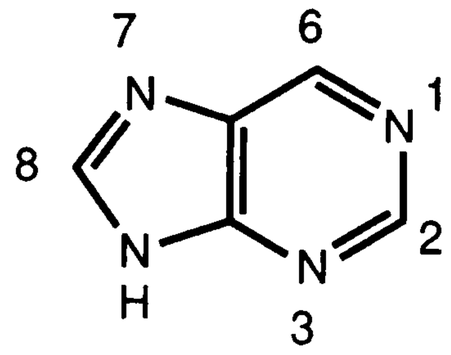


Fig. 2.21 Purine base

The results were in agreement with other workers[114], in that the 2,6-disubstituted purines were found to act as acceptors with the N-deoxyribosyltransferase enzyme but their respective velocities, when compared to the natural C–6 mono-substituted purines, were considerably lower. Of all the purines investigated, 2,6–diaminopurine was found to have the slowest rate of transfer with both 2′-deoxycytidine and 2′,3′-dideoxycytidine and although it was found to transfer on an analytical scale, only a minor amount of product is seen in preparative scale reactions. The positions 1,2,3 and 6 are not essential for binding although large substituents on the 6–position will reduce the rate of reaction, possibly because of steric hindrance.

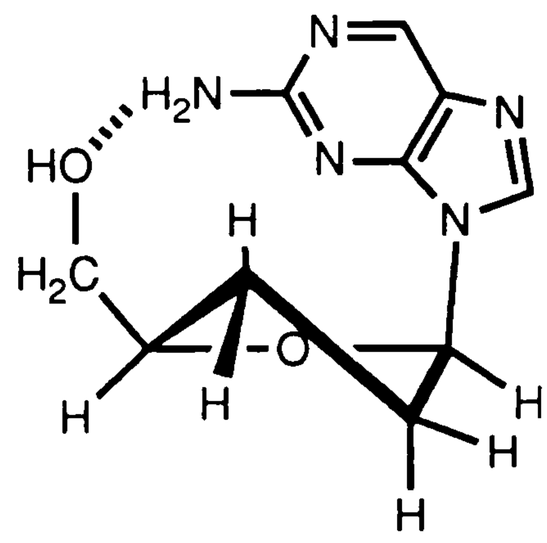


Fig. 2.22 *Syn*-conformation in 9-β-D-2′,3′-dideoxyribofuranosyl-2-aminopurine (**23**).

The successful synthesis of the 2′-deoxy-(**22**) and 2′,3′-dideoxynucleoside (**23**) of 2-aminopurine was demonstrated and the 9-β-D-2′,3′-dideoxyribofuranosyl-2-aminopurine (**23**) was shown to have anti–HIV and anti–SIV activity. The 2′,3′-dideoxynucleoside was unusual in that it lacks an oxygen, nitrogen or chlorine substituent at C–6 in the purine ring and appeared from spectroscopic studies to have the *syn*-conformation, perhaps caused by hydrogen bonding between the exocyclic C5′–OH on the sugar and the C2–NH_2 on the purine base.

2.3: GENERAL MATERIALS AND METHODS

2.3.1: Materials

Chemicals and starting materials were either commercially available or synthesised as described in the text. Chemicals were either purified using literature methods[164], or purchased as the highest available grade. All solvents were dried and distilled before use.

2.3.2: Methods

^{1}H NMR spectra were recorded at 220MHz on a Perkin-Elmer R34 spectrometer, at 250MHZ on a Bruker ACF250 spectrometer, or at 400MHZ on a Bruker WH400 spectrometer. Chemical shifts are given in ppm relative to either tetramethylsilane (TMS) (0.00ppm) or sodium 3-(trimethylsilyl)-1-propane sulphonate (TSS) as internal standards. Multiplicities of ^{1}H NMR signals, where applicable, are abbreviated as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), ddd (doublet of doublet of doublets), dt (doublet of triplets), dq (doublet of quartets), and m (mutliplet).

^{13}C NMR spectra were recorded at 100.62MHz on a Bruker WH400 spectrometer or at 62.89MHz on a Bruker ACF250 spectrometer with $CHCl_3$ and CH_3OD as internal standards.

^{19}F NMR spectra were recorded at 235.192MHz on a Bruker ACF250 spectrometer with $CFCl_3$ as an internal standard.

Long range ^{13}C-^{1}H shift correlation was carried out by a heteronuclear COSY-type experiment, with the use of a composite 180° carbon pulse, and refocussing delays of 3.7ms and 1.85ms.

Nuclear Overhauser enhancement spectra were measured on a solution that had not been deoxygenated and were acquired with an irradiation time of 2 seconds.

Mass spectra were recorded using a Kratos MS80 instrument. Electron impact (EI) spectra were recorded at 70eV and for chemical ionisation (CI) spectra ammonia was used as the carrier gas.

High-Performance Liquid Chromatography (HPLC) analysis was performed on a Waters or Gilson machine. The concentration of nucleosides and bases present in the reaction mixture were determined using reverse phase HPLC on a Techsphere 5C8 column (25cm x 4.6 mm and a precolumn, 5cm x 4.6mm; HPLC Technology Ltd, Macclesfield, Cheshire, UK). The samples were eluted from the column using a mobile phase of acetonitrile and double distilled water at a flow rate of 1.2 ml/min and detected by UV at 254nm.

Unless otherwise stated t.l.c. analyses were run on aluminium plates coated with silica gel (Merck $60_{F\ 254}$, 0.20mm) and eluted in the solvent systems given in the text. Visualisation was achieved by UV or by spraying the plate with 10% H_2SO_4 in ethanol followed by heating to observe the sugars. Compounds were usually purified by flash chromatography on silica gel.

2.3.3: Microorganisms and Media

Lactobacillus leichmannii was obtained from the American Type Culture Collection (ATCC, Rochville, Maryland, USA). Adenosine deaminase E.C.3.5.4.4, Type VI from calf intestinal mucosa, was purchased from Sigma, Poole, Dorset, UK. The growth media MRS Broth[165]was purchased from Oxoid, Basingstoke, Hampshire, UK, and was used as instructed on the bottle. The BioRad protein assay was purchased from BioRad, Watford, Hertfordshire, UK.

2.4: EXPERIMENTAL

2.4.1: Chemical Synthesis of Nucleosides and Bases

2.4.1.1: Synthesis of 3',deoxythymidine (6)

Synthesis of diphenyl sulphite[152]

Thionyl chloride (32.70 ml, 0.17 mol) in dry ether (30 ml) was added dropwise, from a pressure equalised funnel under nitrogen, to phenol (32.44 g, 0.34 mol) in dry ether (150 ml) and dry pyridine (26.30 ml, 0.34 mol) at -5°C with vigorous stirring. The precipitated pyridine hydrochloride (37 g) was filtered off through a sinter funnel and the filtrate was collected and reduced *in vacuo*. The remaining solution was distilled (b.p. 135°C/2mm Hg) to give 34.02 g (86% yield): Rf=0.65 (diethyl ether/petroleum ether (40-60), 1/4); MS (EI) m/z (%) 77 (100), 94 (48), 141 (97), 169 (5), 234 (43, $M^{+\cdot}$),

Synthesis of 2,3′-anhydrothymidine (4)[151]

Thymidine (5.0 g, 20.6 mmol), diphenyl sulphite (19.34 g, 82.6 mmol), 1-methylimidazole (0.34 ml, 4.27 mmol) and N,N-dimethylacetamide (50 ml) were stirred at 156°C for 1 h. The reaction was then cooled to 0°C and poured, with stirring, into a mixture of triethylamine (50 ml) and water (90 ml) at 0°C. The solution warmed to room temperature after 40 min and was then washed with chloroform (4 x 50 ml). The remaining aqueous layer was concentrated under reduced pressure and the viscous oil obtained was dissolved in absolute ethanol (2 x 30 ml), and the solution was re-evaporated. The residue was triturated with ether (3 x 50 ml) and finally CH_2Cl_2 (50 ml) was added and left overnight to crystallise the product. The colourless precipitate was collected by filtration, washed with CH_2Cl_2 (2 x 10 ml) and lyophilised to give 2.62 g (56.6% yield): Rf=0.13 ($MeOH/CH_2Cl_2$, 5/95); ^1H NMR (CD_3OD) δ 1.95 (3H, d, J=1.11Hz, Me), 2.55-2.72 (2H, m, $H_{2'a\ and\ b}$), 3.73 (2H, d, J=6.33Hz, $H_{5'a\ and\ b}$), 4.36 (1H, ddd, J=2.23, 2 x 6.35Hz, $H_{4'}$), 5.36 (1H, d, J=2.22Hz, $H_{3'}$), 5.93 (1H, d, J=2.97Hz, $H_{1'}$), 7.62 (1H, d, J=1.11Hz, H_6); ^{13}C NMR (CD_3OD) δ 13.96 (C_5-Me), 34.52 ($C_{2'}$), 61.44 ($C_{5'}$), 79.25 ($C_{3'}$), 87.26 ($C_{1'}$), 89.29 ($C_{4'}$), 118.66 (C_5), 139.07 (C_6), 153.17 (C_2), 167.12 (C_4); MS (EI) m/z (%) 55 (54), 69 (78), 81 (100), 98 (53), 110 (30), 126 (53), 149 (25), 206 (9), 224 (21, $M^{+\cdot}$); (CI) m/z (%) 127 (14), 225 [10, $(M+H)^+$].

Synthesis of 1-(2',3'-dideoxy-β-D-glycero-pento-2'-enofuranosyl)thymine (5)[145]

To a solution of *t*-BuOK (0.90 g, 8.0 mmol) in DMSO (20 ml) was added 2,3′-anhydrothymidine (1.68 g, 7.5 mmol). The reaction mixture was stirred under nitrogen at room temperature for 1 h and then warmed to

60°C for a further 1 h. Water (10 ml) was added and the mixture was neutralised from pH 10 to pH 7 with dilute acetic acid (~ 3 ml) to produce a gelatinous precipitate which was filtered, washed with water (2 x 10 ml) and lyophilised. The solid was purified by flash chromatography on silica gel($MeOH/CH_2Cl_2$, 1/9) to remove DMSO and unreacted starting material to give 1.21 g (72% yield): Rf=0.44 ($MeOH/CH_2Cl_2$, 1/9); ^1H NMR (CD_3OD) δ 1.88 (3H, d, J=1.13Hz, Me), 3.76 (1H, dd, J=3.10, 12.5Hz, $H_{5'a}$), 3.83 (1H, dd, J=3.03, 12.5Hz, $H_{5'b}$), 4.88 (1H, brs, $H_{4'}$), 5.95 (1H, dq, J=1.88, 5.96Hz, $H_{2'}$), 6.44 (1H, dt, J=1.67, 5.95Hz, $H_{3'}$), 6.99 (1H, q, J=1.86, 3.35Hz, $H_{1'}$), 7.79 (1H, d, J=1.12Hz, H_6); ^{13}C NMR (CD_3OD) δ 12.40 (C_5-Me), 63.81 ($C_{5'}$), 88.98 ($C_{4'}$), 91.06 ($C_{1'}$), 111.21 (C_5), 127.32 ($C_{2'}$), 135.96 ($C_{3'}$), 138.92 (C_6), 152.90 (C_2), 166.59 (C_4); MS (CI) m/z (%) 99 (20), 127 (100), 155 (13), 193 (8), 207 (15), 225 [20, $(M+H)^+$].

Synthesis of 3′,5′-dimethanesulphonylthymidine (2)

Thymidine (5.0 g, 20.0mmol) and DMAP (4 mg) were dissolved in dry pyridine (60 ml). Methanesulphonyl chloride (6.0 ml, 50.0mmol) was added to the cooled solution at 0°C under nitrogen. The reaction mixture was stirred overnight at room temperature and then poured onto ice-water (500 ml), with vigorous stirring. The precipitate was collected by filtration through a glass sinter funnel, and washed with water (10 ml). The white precipitate was dissolved in acetone, filtered and reduced *in vacuo* before being lyophilised to give 7.6 g (92% yield): Rf=0.54 ($MeOH/CH_2Cl_2$, 15/85); ^1H NMR (CD_3OD) δ 1.93 (3H, d, J=1.08Hz, Me), 2.51 (1H, ddd, J=2 x 6.92, 14.8Hz, $H_{2'b}$), 2.64 (1H, ddd, J=2.28, 6.06, 14.6Hz, $H_{2'a}$), 3.16 (3H, s, SO_2Me), 3.19 (3H, s, SO_2Me), 4.48 (1H, ddd appears as quintet, J=2.97, 3.87, 6.84Hz, $H_{4'}$), 4.56 (2H, m, $H_{5'a \text{ and } b}$). 5.37 (1H, ddd, J=2 x 2.82, 6.80Hz, $H_{3'}$), 6.32 (1 H, dd, J=6.09, 8.07Hz, $H_{1'}$), 7.47 (1H, d, J=1.17Hz, H_6); MS

(EI) m/z (%) 55 (100), 70 (80), 96 (63), 126 (55, thymine$^{+\cdot}$); (CI) m/z (%) 127 [67, (thymine+H)$^+$], 144 (4), 207 (6), 303 (8), 399 [2, (M+H)$^+$].

Synthesis of 1-(2'-deoxy-3′,5′-epoxy-β-D-*threo*-pentofuranosyl)thymine (3)[146]

A solution of 3′,5′-dimesylthymidine (7.5 g, 0.02 mol) in water (300 ml) containing sodium hydroxide (56 ml, 1.0 N) was refluxed for 4 h under nitrogen. The reaction mixture was cooled to room temperature and neutralised with HCl (19 ml, 1.0 N) before being concentrated *in vacuo*. The white crystals were dissoved in chloroform (30 ml) and the impurities were removed by filtration. The solution was concentrated *in vacuo* before being lyophilised to give 3.2 g (75% yield): Rf=0.41 ($MeOH/CH_2Cl_2$, 1/9); ^1H NMR (CD_3OD) δ 1.92 (3H, d, J=1.12Hz, Me), 2.53-2.62 (2H, m, J= 3.00, 5.41Hz, $H_{2'a \text{ and } b}$), 4.16 (1H, dd, J=1.73, 8.16Hz, $H_{4'}$), 4.82 (1H, dd, J=3.97, 8.20Hz, $H_{5'a}$), 4.97 (1H, m, J=1.88, 4.01Hz, $H_{5'b}$), 5.58 (1H, ddd appears as q, J=3.65, 6.25Hz, $H_{3'}$), 6.64 (1H, dd appears as t, J=2 x 5.34Hz, $H_{1'}$), 8.16 (1H, d, J=1.18Hz, H_6); ^{13}C NMR (CD_3OD) δ 13.05 (C_5-Me), 38.90 ($C_{2'}$), 77.19 ($C_{5'}$), 82.21 ($C_{3'}$), 89.05 ($C_{4'}$), 90.40 ($C_{1'}$), 112.33 (C_5), 138.58 (C_6), 153.29 (C_2), 166.34 (C_4); MS (EI) m/z (%) 55 (40), 69 (90), 81 (1), 99 (100), 110 (16), 126 (75), 224 (23, M$^{+\cdot}$); (CI) m/z (%) 55 (1), 64 (2), 81 (4), 99 (4), 127 (18), 144 (2), 225 [100, (M+H)$^+$].

Synthesis of 1-(2',3'-dideoxy-β-D-glycero-pento-2'-enofuranosyl)thymine (5)[146]

To a solution of *t*-BuOK (3.0 g, 27.0 mmol) in DMSO (15 ml) was added 3,5′-cyclothymidine (3.0 g, 13.0 mmol). The reaction mixture was stirred under nitrogen at room temperature for 3 h. The mixture was neutralised

from pH 10 to pH 7 with dilute acetic acid (~ 9 ml) to produce a gelatinous precipitate which was filtered, washed with water (2 x 10 ml) and lyophilised. The residue was triturated with hot acetone (5 x 20 ml) and the salts were filtered off. The filtrate was concentrated *in vacuo* to produce a brown residue which was dissolve in absolute ethanol (10 ml) and benzene (25 ml) and concentrated on a hot plate to 5 ml. This procedure was continued until a slight turbidity was evident (4 times) and then the solution was left at 4°C until the product crystallised. The product was collected by filtration and recrystallised from ethanol and benzene to give 1.42 g (47% yield): Rf=0.38 ($MeOH/CH_2Cl_2$, 1/9); ^1H NMR (CD_3OD) δ 1.88 (3H, d, J=1.13Hz, Me), 3.76 (1H, dd, J=3.10, 12.5Hz, $H_{5'a}$), 3.83 (1H, dd, J=3.03, 12.5Hz, $H_{5'b}$), 4.88 (1H, brs, $H_{4'}$), 5.95 (1H, dq, J=1.88, 5.96Hz, $H_{2'}$), 6.44 (1H, dt, J=1.67, 5.95Hz, $H_{3'}$), 6.99 (1H, quintet, J=1.86, 3.35Hz, $H_{1'}$), 7.79 (1H, d, J=1.12Hz, H_6); ^{13}C NMR (CD_3OD) δ 12.40 (C_5-Me), 63.81 ($C_{5'}$), 88.98 ($C_{4'}$), 91.06 ($C_{1'}$), 111.21 (C_5), 127.32 ($C_{2'}$), 135.96 ($C_{3'}$), 138.92 (C_6), 152.90 (C_2), 166.59 (C_4); MS (EI) m/z (%) 55 (60), 69 (100), 99 (46), 126 (11), 150 (5), 193 (15); (CI) m/z (%) 99 (20), 127 (100), 155 (13), 193 (8), 207 (15), 225 [20, $(M+H)^+$].

Synthesis of 3′-deoxythymidine (6)[146]

A solution of 1-(2,3-dideoxy-β-D-glycero-pento-2-enofuranosyl)thymine (1.20 g, 5.36 mmol) in absolute ethanol (100 ml) containing 10% palladium-charcoal catalyst (0.2 g) was stirred under 1 atm of hydrogen at room temperature for 3 h. The solution was filtered through a celite pad and the filtrate was evaporated to dryness *in vacuo* to give 0.81g (67% yield) of a white solid: Rf=0.61 ($MeOH/CH_2Cl$, 1/4); ^1H NMR (CD_3OD) δ 1.78 (3H, d, J=1.04Hz, Me), 1.74-2.36 (4H, m, $H_{2'a \text{ and } b}$ & $H_{3'a \text{ and } b}$), 3.58 (1H, dd, J=3.72, 12.2Hz, $H_{5'a}$), 3.78 (1H, dd, J=2.96, 12.2Hz, $H_{5'b}$), 4.03 (1H, dddd appears as septet, J=2 x 3.41, 2 x 7.17Hz, $H_{4'}$), 5.95 (1 H, dd, J=3.12, 6.51Hz,

$H_{1'}$), 8.07 (1H, d, J=1.14Hz, H_6); ^{13}C NMR (CD_3OD) δ 12.53 (C_5-Me), 25.91 ($C_{2'}$), 33.37 ($C_{3'}$), 63.71 ($C_{5'}$), 83.22 ($C_{4'}$), 87.32 ($C_{1'}$), 110.86 (C_5), 138.45 (C_6), 152.40 (C_2), 166.58 (C_4); HRMS (EI) calc ($C_{10}H_{14}N_2O_4$) 226.0953, found 226.0953.

2.4.1.2: Synthesis of 2′,3′-dideoxycytidine (11)

Synthesis of 5′-O-(*tert*-butyldimethylsilyl)-cytidine (7)

Cytidine (5.0g, 20mmol) and imidazole (0.26g, 48mmol) in dry pyridine (30ml) was cooled in an ice-water bath followed by the addition of *tert*-butyldimethylsilyl chloride (3.62g, 24mmol). The mixture was stirred under nitrogen overnight at room temperature and then poured into water and extracted with CH_2Cl_2 (2 x 50ml). The organic layer was washed with water (2 x 30ml) and aqueous sodium chloride (30ml), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 2/98) to give 5.71g (80% yield) of a white solid: ^1H NMR ($CDCl_3$) δ 0.09 (6H, s, Me_2Si), 0.09 (9H, s, Me_3CSi), 3.75-4.08 (5H, m, $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'a\ and\ b}$), 5.07 (1H, brs, OH), 5.59 (1H, d, J=4.7Hz, OH), 5.69 (1H, d, J=7.6Hz, H_5), 5.76 (1H, dd appears as t, J=6.12Hz, $H_{1'}$), 7.12 (2H, s, NH_2), 8.31 (1H, d, J=7.6Hz, H_6).

Synthesis of 5′-O-(*tert*-butyldimethylsilyl)-2′,3′-bis-O-phenoxythiocarbonyl cytidine (8)

A solution of phenyl chlorothionoformate (7.3g, 42.0mmol) in dry CH_2Cl_2 (20ml) was added dropwise to a stirred solution of the silyl protected cytidine (5.0g, 14.0mmol) and 4-(dimethylamino)pyridine (6.83g, 56.0mmol) in dry CH_3CN (100ml) at -20°C. The solution was stirred at

room temperature for 6 h and then the solvent was evaporated and the residue was purified by flash chromatography on silica gel (CH_2Cl_2/EtOAc, 10/1) to give 7.75g (88% yield) of a pale yellow solid: ^1H NMR ($CDCl_3$) δ 0.04 (6H, s, Me_2Si), 0.85 (9H, s, Me_3CSi), 3.83 (1H, dd, J=4.01, 12.3Hz, $H_{5'a}$), 3,92 (1H, dd, J=3.02, 12.3Hz, $H_{5'b}$), 4.56 (1H, ddd appears as q, J=3 x 5.6Hz, $H_{4'}$), 5.72 (1H, d, J=7.6Hz, H_5), 6.14-6.42 (3H, m, $H_{1'}$, $H_{2'}$, $H_{3'}$), 7.14 (2H, s, NH_2), 7.20-7.55 (10H, m, 2 x Ph), 8.13 (1H, d, J=7.6Hz, H_6).

Synthesis of 5′-O-(*tert*-butyldimethylsilyl)-2′,3′-didehydro-2′,3′-dideoxy cytidine (9)

A refluxing solution of (8) (7.0g, 11.0mmol) and azobis(isobutyronitrile) (0.83g, 5.6mmol) in dry toluene (100ml) was treated with a solution of tri-*n*-butyltin hydride (9.77g, 33.3mmol) in dry toluene. The solution was stirred under nitrogen at reflux for 3 h before the solvent was removed *in vacuo*. The residue was purified by flash chromatography on silica gel (MeOH/CH_2Cl_2, 2/98) to give 2.56g (72% yield) of a white solid: ^1H NMR ($CDCl_3$) δ 0.04 (6H, s, Me_2Si), 0.83 (9H, s, Me_3CSi), 3.71 (1H, dd, J=4.07, 12.2Hz, $H_{5'a}$), 3.89 (1H, dd, J=2.97, 12.2Hz, $H_{5'b}$), 4.89 (1H, ddd, J=1.95, 3.01, 3.99Hz, $H_{4'}$), 5.21 (1H, d, J=7.4Hz, H_5), 6.03 (1H, dd, J=2.12, 6.15Hz, $H_{2'}$), 6.38 (1H, dd, J=1.97, 6.15Hz, $H_{3'}$), 6.78 (1H, d, J=2.08Hz, $H_{1'}$), 7.16 (2H, s, NH_2), 8.12 (1H, d, J=7.3Hz, H_6).

Synthesis of 2′,3′-didehydro-2′,3′-dideoxycytidine (10)

The silylated 2′,3′-didehydro-2′,3′-dideoxycytidine (9) (2.50g, 7.7mmol) in dry THF (30ml) was cooled in an ice-water bath before the addition of a 1.0M solution of tetra-*n*-butylammonium fluoride in THF (15ml, 15mmol). The mixture was stirred at room temperature for 1 h before the

solvent was removed *in vacuo*. The resulting pale yellow syrup was purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 5/95) to give 1.48g (92% yield) of a pale yellow solid: ^1H NMR (DMSO-d_6) δ 3.42 (1H, m, $H_{5'a}$), 3.60 (1H, m, $H_{5'b}$), 4.74 (1H, ddd, J=1.12, 2.81, 3.94Hz, $H_{4'}$), 4.87 (1H, t, J=5.48Hz, OH), 5.67 (1H, d, J=7.3Hz, H_5), 5.83 (1H, dd, J=1.17, 5.92Hz, $H_{2'}$), 6.35 (1H, dd, J=1.09, 5.94Hz, $H_{3'}$), 6.90 (1H, d, J=1.21Hz, $H_{1'}$), 7.11 (2H, s, NH_2), 7.71 (1H, d, J=7.3Hz, H_6).

Synthesis of 2′,3′-dideoxycytidine (11)

2′,3′-Didehydro-2′,3′-dideoxycytidine (1.40g, 6.7mmol) in ethanol (100ml) was hydrogenated at 30psi in the presence of 10% Pd/C (200mg) for 2 h. The reaction mixture was filtered through a celite pad and the filtrate was concentrated *in vacuo*. The crude product was purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 5/95) to give 1.24g (88% yield) of a white solid: ^1H NMR (CD_3OD) δ 1.86-2.50 (4H, 4 x dddd appears as dddd and m, J=6.87, 6.72, 7.81, 10.04Hz, $H_{2'a \text{ and } b}$, $H_{3'a \text{ and } b}$), 3.73 (1H, dd, J=4.06, 12.2Hz, $H_{5'a}$), 3.91 (1H, dd, J=2.28, 12.2Hz, $H_{5'b}$), 4.19 (1H, dddd, J=3.21, 4.10, 6.33, 6.70Hz, $H_{4'}$), 5.91 (1H, d, J=8.10Hz, H_5), 6.06 (1H, dd, J=3.36, 6.67Hz, $H_{1'}$), 8.15 (1H, d, J=8.10Hz, H_6); ^{13}C NMR (CD_3OD) δ 25.91 ($C_{2'}$), 33.53 ($C_{3'}$), 63.73 ($C_{5'}$), 83.53 ($C_{4'}$), 87.73 ($C_{1'}$), 102.02 (C_5), 142.73 (C_6), 152.32 (C_2), 166.53 (C_4); HRMS (EI) calc ($C_9H_{13}N_3O_3$) 211.0957, found 211.0954.

2.4.1.3: Synthesis of N^6-methoxyadenine (12)

a) 6-Chloropurine (1.0g, 7.4mmol), methoxyamine hydrochloride (6.2g, 74mmol) and calcium carbonate (14.83g, 148mmol) in absolute ethanol (100ml) were stirred at reflux for 4 h under nitrogen. The solvent was removed *in vacuo* and the residue was purified by ion-exchange

chromatography on Dowex 50 H^+. The product was eluted with increasing molarities of ammonium hydroxide solution from 0.1M to 0.5M to give 760mg (62 % yield) of a pale yellow solid: UV λ_{max}=201nm (41300), 270nm (12500); ^1H NMR (D_2O) δ 3.79 (3H, s, OCH_3), 7.77 (1H, brs, H_2), 7.92 (1H, brs, H_8); ^{13}C NMR (D_2O) δ 70.13 (OCH_3), 120.93 (C_5), 142.41 (C_8), 148.75 (C_4), 158.00 (C_2), 158.50 (C_6); MS (EI) m/z(%) 120 (44), 135 (100), 150 (14), 165 (44, $M^{+\cdot}$); (CI) m/z (%) 136 (100, adenine$^+$), 166 [45, (M+ H)$^+$].

b) To a solution of adenine (0.5g, 3.7mmol) in distilled water (10ml) was added a solution of *m*-chloroperbenzoic acid (1.92g, 11.1mmol) in dioxane (20ml). The mixture was stirred at room temperature for 3 h before the solvent was removed *in vacuo* and the residue was washed with acetone. The white precipitate was collected by filtration from the yellow filtrate. The crude adenine 1-oxide was recrystallised from ethanol to give 0.54g (96% yield) of white crystals. The adenine 1-oxide (0.47g, 3.1mmol) was dissolved in dry DMF (10ml) and methyl iodide (1.99g, 0.87ml, 14.0mmol) was added. The mixture was stirred under nitrogen at room temperature for 3 h but there was still some solid material so the mixture was heated at 60°C for a further 2 h. To the resulting yellow solution was added ether (30ml) and the precipitate was filtered off. The crude 1-methoxyadenine hydroiodide was dissolved in water (20ml) and the pH was adjusted to 8 by the addition of triethylamine before being heated at 60°C for 8 h. The solvent was removed *in vacuo* and the residue was crystallised from hot water to give 180mg (35% yield) of pale pink crystals: data as above.

c) Hydrolysis of N^6-methoxy-2′-deoxyadenosine

The N^6-methoxy-2′-deoxyadenosine (180mg, 0.64mmol) was dissolved in distilled water (10ml) and Dowex 50 H^+ (5g) was added. The mixture was stirred for 2 h before the Dowex was filtered off and washed with water (2 x

20ml). The 2-deoxyribose was in the aqueous layer while the required N6-methoxyadenine was bound to the Dowex. The base was eluted from the Dowex with 0.5N ammonium hydroxide solution (20ml) and the solution was evaporated *in vacuo* to give 75mg (71% yield) of the base: data as above.

2.4.1.4: Synthesis of N^6-methoxy-2′-deoxyadenosine (15)[156]

Synthesis of 2′-deoxyadenosine 1-oxide (13)

To a solution of 2′-deoxyadenosine (1.0g, 4.0mmol) in distilled water (20ml) was added a solution of *m*-chloroperbenzoic acid (2.8g, 16mmol) in dioxane (30ml). The mixture was stirred at room temperature for 3 h. The solvent was removed *in vacuo* and the residue was washed with acetone. The resulting precipitate was collected by filtration and the crude product was recrystallised from ethanol (~15ml) to give 0.82g (77% yield) of a white solid: ^{1}H NMR (DMSO-d_6) δ 2.30 (1H, ddd, J=3.69, 6.33, 13.3Hz, $H_{2'a}$), 2.70 (1H, ddd appears as quintet, J=2x6.05, 13.2Hz, $H_{2'b}$), 3.50 (1H, dd, J= 4.49, 11.6Hz, $H_{5'a}$), 3.59 (1H, dd, J=4.65, 11.7Hz, $H_{5'b}$), 3.85 (1H, ddd appears as q, J=3x4.57Hz, $H_{4'}$), 4.40 (1H, ddd appears as q, J=2x3.31, 6.02Hz, $H_{3'}$), 4.98 (1H, brs, NH), 5.36 (1H, brs, NH), 6.32 (1H, dd appears as t, J=2x6.73Hz, $H_{1'}$), 8.51 (1H, s, H_2), 8.63 (1H, s, H_8); ^{13}C NMR (DMSO-d_6) δ 39.4 ($C_{2'}$), 61.6 ($C_{5'}$), 70.6 ($C_{3'}$), 83.6 ($C_{1'}$), 88.0 ($C_{4'}$), 128.0 (C_5), 141.6 (C_8), 142.4 (C_4), 143.3 (C_2), 148.4 (C_6).

Synthesis of 1-methoxy-2′-deoxyadenosine hydroiodide (14)

2′-Deoxyadenosine 1-oxide (0.70g, 2.6mmol) was dissolved in dry DMF (10ml) and methyl iodide was added (1.67g, 0.73ml, 11.7mmol). The mixture was stirred under nitrogen at room temperature overnight. The required product was precipitated by the addition of ether (50ml). The crude 1-methoxy-2′-deoxyadenosine hydroiodide was filtered off to give 550mg (75% yield) of a white crystalline precipitate: ^{1}H NMR (D_2O) δ 2.67 (1H, m, $H_{2'a}$), 2.85 (1H, m, $H_{2,b}$), 3.82 (2H, 2 x dd, $H_{5'a\ and\ b}$), 4.17 (1H, q, $H_{4'}$), 4.34 (3H, s, OCH_3), 4.68 (1H, m, $H_{3'}$), 6.54 (1H, t, $H_{1'}$), 8.62 (1H, s, H_2), 9.03 (1H, s, H_8).

Synthesis of N^6-methoxy-2′-deoxyadenosine (15)

The crude 1-methoxy-2′-deoxyadenosine hydroiodide (500mg, 1.87mmol) was dissolved in water (15ml) and the pH was adjusted to 8 by the addition of triethylamine before being heated at 60°C for 6 h. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 5/95), to give 370mg (74% yield) of pale pink crystals: ^{1}H NMR (CD_3OD) δ 2.43 (1H, ddd, J=2.92, 6.09, 13.5Hz, $H_{2'a}$), 2.76 (1H, ddd, J=5.89, 7.83, 13.6Hz, $H_{2'b}$), 3.75 (1H, dd, J=3.65, 12.2Hz, $H_{5'a}$), 3.85 (1H, dd, J=3.25, 12.2Hz, $H_{5'b}$), 3.89 (3H, s, OCH_3), 4.07 (1H, ddd, J=3x3.37Hz, $H_{4'}$), 4.58 (1H, ddd, J=2x2.77, 5.69Hz, $H_{3'}$), 6.39 (1H, dd, J=6.08Hz, $H_{1'}$), 7.82 (1H, brs, H_2), 8.15 (1H, s, H_8); ^{13}C NMR (CD_3OD) δ 41.8 ($C_{2'}$), 62.5 (OCH_3), 63.5 ($C_{5'}$), 72.9 ($C_{3'}$), 86.7 ($C_{1'}$), 89.7 ($C_{4'}$),93.1 (C_5), 95.1 (C_8), 120.2 (C_4), 139.5 (C_2), 147.1 (C_6); MS (EI) m/z (%) 281 (1.3, M^+), 250 (1.2, $M^{+\cdot}-OCH_3$), 236 (1.1, $M^{+\cdot}-NOCH_3$), 221 (1.7), 205 (0.8), 192 (10.3), 165 (27.8, N^6-methoxyadenine$^{+\cdot}$), 135 (100, adenine$^{+\cdot}$); (CI) = 282 [6.2, ($M+H^+$)], 252 (54.9), 166 (6.9), 136 (47.2).

2.4.1.5: Synthesis of N^4-methoxy-5-methylcytidine (17)[159]

Synthesis of 3′,5′-di-*tert*-butylmethylsilylthymidine (16)

Thymidine (2.42g, 10mmol) and imidazole (2.02g, 30mmol) were dissolved in DMF (20ml) and *tert*-butyldimethylsilyl chloride (4.5g, 30mmol) was added. The mixture was stirred at room temperature for 3 h before the volume was reduced to 5ml *in vacuo*. The solution was dissolved in diethyl ether (40ml), washed with saturated aqueous sodium chloride (3 x 40ml), dried over anhydrous $MgSO_4$ and filtered. The filtrate was concentrated *in vacuo* to give 4.15g (88% yield) of the crude product as a white solid: Rf = 0.67 ($MeOH/CH_2Cl_2$, 1/9); ^1H NMR (CD_3OD) δ 0.05 (6H, d, J=2.79Hz, Me_2Si), 0.09 (6H, d, J=1.06Hz, Me_2Si), 0.87 (9H, s, Me_3Si), 0.90 (9H, s, Me_3Si), 1.98 (1H, ddd, J=6.06, 7.91, 13.1Hz, $H_{2'b}$), 2.22 (1H, ddd, J=2.63, 5.81, 13.1Hz, $H_{2'a}$), 3.73 (1H, dd, J=2.42, 11.37Hz, $H_{5'a}$), 3.84 (1H, dd, J=2.58, 11.38Hz, $H_{5'b}$), 3.91 (1H, ddd appears as q, J=3 x 2.49, $H_{4'}$), 4.38 (1H, ddd appears as quintet, J=2 x 2.56, 5.99Hz, $H_{3'}$), 6.32 (1H, dd, J=5.83, 7.90Hz, $H_{1'}$), 7.45 (1H, d, J=1.22Hz, H_6), 9.25 (1H, brs, NH); ^{13}C NMR (CD_3OD) δ -5.60, -5.52, -4.99, -4.78 (2 x Me_2Si), 12.40 (C_5-Me), 17.86, 18.26, 25.60, 25.70 (2 x Me_3Si), 41.24 ($C_{2'}$), 62.82 ($C_{5'}$), 72.08 ($C_{3'}$), 84.67 ($C_{1'}$), 87.66 ($C_{4'}$), 110.70 (C_5), 135.33 (C_6), 150.26 (C_2), 163.85 (C_4).

Synthesis of N^4-methoxy-5-methylcytidine (17)

Methoxylamine hydrochloride (0.70g, 8.4mmol) was suspended in dry CH_3CN (10ml) at 0°C. $POCl_3$ (0.2ml) and triethylamine (1.7ml) were added slowly and the mixture was stirred for 1h. 3′,5′-Di-*tert*-butylmethylsilylthymidine (0.27g, 5.7mmol) dissolved in CH_3CN (3ml) was added over 15 min and the resulting solution was stirred at room

temperature overnight. The solution was filtered, diluted with ethyl acetate (20ml), washed with saturated aqueous $NaHCO_3$ (20ml) and saturated aqueous NaCl (2 x 20ml). The organic layer was dried over $MgSO_4$ and filtered. The filtrated was evaporated *in vacuo* to give the crude product. The pale yellow solid was purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 5/95) to give 1.41g (91% yield) of the desilylated product: Rf = 0.32 ($MeOH/CH_2Cl_2$, 1/9); ^1H NMR (CD_3OD) δ 1.79 (1H, d, J=1.11Hz, Me), 2.17 (1H, ddd, J=3.46, 6.48, 13.7Hz, $H_{2'b}$), 2.33 (1H, m, J=7.10, 14.0Hz, $H_{2'a}$), 3.75 (1H, dd, J=3.47, 11.92Hz, $H_{5'a}$), 3.82 (1H, dd, J=3.25, 11.94Hz, $H_{5'b}$), 3.83 (3H, s, OMe), 3.91 (1H, ddd appears as q, J=3 x 3.46Hz, $H_{4'}$), 4.48 (1H, ddd appears as quintet, J=2 x 3.40, 6.72Hz, $H_{3'}$), 6.09 (1H, dd appears as t, J=7.05Hz, $H_{1'}$), 6.60 (1H, d, J=1.23Hz, H_6), 8.23 (1H, brs, NH); ^{13}C NMR (CD_3OD) δ 12.60 (C_5-Me), 38.97 ($C_{2'}$), 61.67 (OMe), 62.42 ($C_{5'}$), 71.47 (C_3), 85.93 ($C_{1'}$), 86.32 ($C_{4'}$), 107.19 (C_5), 127.71 (C_6), 144.38 (C_2), 149.35 (C_4); MS (EI) m/z (%) 124 (29), 155 (100), 271 (6, M^+); (CI) m/z (%) 81 (1.9), 98 (2.7), 116 (3.0), 126 (6.6), 156 (4.3), 182 (1.3), 217 (0.4), 242 (1.1), 272 [3, $(M+H)^+$]; HRMS (EI) calcd ($C_{11}H_{17}N_3O_5$) 271.1169, found 271.1185.

2.4.1.6: Synthesis of N^4-methoxy-5-methylcytosine (18)[159]

Methoxylamine hydrochloride (1.40g, 16.8mmol) was suspended in dry CH_3CN (20ml) at 0°C. $POCl_3$ (0.4ml) and triethylamine (4.4ml) were added to the suspension slowly and the mixture was stirred for 0.5 h before thymine (0.142g, 1.13mmol) in CH_3CN (20ml) was added over a period of 10 min. The solution was stirred at room temperature overnight and then heated for 5 h at 60°C. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 3/97) to give 126mg (72% yield) of a white solid: Rf = 0.61 ($MeOH/CH_2Cl_2$, 1/9); ^1H NMR (CD_3OD) δ 1.79 (3H, d, J=1.33Hz, Me), 3.86 (3H, s, OMe), 6.57 (1H,

d, J=1.33Hz, H_6); ^{13}C NMR (CD_3OD) δ 12.63 (C_5-Me), 61.82 (OMe), 107.90 (C_5), 129.83 (C_6), 145.20 (C_2), 150.70 (C_4); MS (EI) m/z (%) 58 (27.3), 86 (100), 101 (22.4), 155 (4.5, M^+); (CI) m/z (%) 86 (10), 102 (62), 156 (19, $(M+H)^+$); HRMS (EI) calcd ($C_6H_9N_3O_2$) 155.0695, found 155.0699.

2.4.2: Enzymatic Synthesis of Nucleoside Analogues

2.4.2.1: Purification of nucleoside N-deoxyribosyltransferases (E. C. 2.4.2.6.) from *Lactobacillus leichmannii*

Lactobacillus leichmannii ATCC 4797 was grown in a MRS (DeMan-Rogosa-Sharpe) medium (52gl^{-1}) previously sterilised by autoclaving at 120°C. The medium was inoculated with a 24 h starter culture (1%) grown in the same medium and incubated at 37°C without aeration or agitation. The bacterial suspension was harvested by centrifugation (10 000g) for 15 min at 4°C. The pellet produced was washed twice with 0.02M PIPES buffer (pH 6.5) containing 0.02% (w/v) sodium azide, and the cell paste stored at -20°C. Thawed cells were disrupted by three passes through a French pressure cell (20 000 psi) keeping the temperature below 10°C, and the cell debris was removed by centrifugation (20 000g) for 30min at 4°C. Cell-free extracts were dialysed overnight against 100 volumes of 0.02M PIPES buffer (pH 6.5) containing 0.02% (w/v) sodium azide. The final preparation could be stored at -20°C for up to 3 months without significant loss of activity.

2.4.2.2: Protein Determination

The concentration of protein was estimated using the dye-binding BioRad protein assay according to the method of Bradford[166]. The colorimetric property of Coomassie blue G-250 was employed, where the absorbance maximum of the dye changes from 465nm to 595nm when binding to protein occurs. Bovine serum albumin was used as the standard.

2.4.2.3: Definition of Unit and Specific Activity

One unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1.0 μmol of product (2′-deoxyadenosine) formed in 1 min in the assay conditions from 2′-deoxycytidine and adenine. Specific acitivity was defined as units per milligram of protein.

2.4.2.4: Standard Assay of N-deoxyribosyltransferase

The standard reaction assay mixture contained 2′-deoxycytidine (1.5 mM), as the donor nucleoside, and adenine (0.5 mM), as the acceptor base, in citrate buffer (50 mM, pH 6.0). The reactions were initiated by the addition of a volume of the crude enzyme preparation equivalent to 50μg/ml of protein. The final volume of the reaction mixture was 0.5 ml and it was incubated at 40°C with no shaking. At intervals of 0, 5, 10, 15, 20, and 30 min a 20 μl aliquot was removed from the reaction mixture and applied to an HPLC column. The rate of deoxyribosyl transfer from the pyrimidine nucleoside to the purine base was measured by following the formation of 2′-deoxyadenosine. The concentration of nucleosides and bases present in the reaction mixture were determined using reverse phase HPLC on a Techsphere 5C8 column (25cm x 4.6 mm and a precolumn, 5cm x 4.6mm).

The samples were eluted from the column using a mobile phase of acetonitrile and double distilled water (5/95) at a flow rate of 1.2 ml/min and detected by UV at 254nm.

2.4.2.5: Comparison of Glycosyl Donors

Four standard assay reactions were set up (as described before):

a) 2′-deoxycytidine as the donor nucleoside and adenine as the acceptor base;

b) thymidine as the donor nucleoside and adenine as the acceptor base;

c) 2′-deoxycytidine as the donor nucleoside and 2-aminopurine as the acceptor base;

d) thymidine as the donor nucleoside and 2-aminopurine as the acceptor base.

The rate of each reaction was followed as described in the standard assay method by reverse phase HPLC. The same set of reactions were also investigated using 2′,3′-dideoxycytidine and 2′,3′-dideoxythymidine as the donor nucleosides.

2.4.2.6: Synthesis of N^6-methoxy-2′-deoxyadenosine (15)

N^6-Methoxyadenine (30mg, 0.18mmol) and 2′-deoxycytidine (144mg, 0.55mmol) were dissolved in citrate buffer (15ml, 10mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (1.5ml, 10%). The crude N-deoxyribosyltransferase extract (1ml, 15.8mg ml^{-1} protein, 7U) was added and the mixture was incubated for 10 days at 40°C. The progress of the reaction was followed by reverse phase HPLC analysis (Techsphere 5C8

column, 25cm x 4.6mm, elution with 6% CH_3CN/10mM NH_4OAc). When the reaction had reached equilibrium, the mixture was lyophilised and the residue was purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 5/95) to give 37mg (72% yield) of white crystals: Rf = 0.18 ($MeOH/CH_2Cl_2$, 1/9); ^1H NMR (CD_3OD) δ 2.43 (1H, ddd, J=2.92, 6.09, 13.5Hz, $H_{2'b}$), 2.76 (1H, ddd, J=5.89, 7.83, 13.6Hz, $H_{2'a}$), 3.75 (1H, dd, J=3.65, 12.2Hz, $H_{5'a}$), 3.85 (1H, dd, J=3.25, 12.2Hz, $H_{5'b}$), 3.89 (3H, s, OCH_3), 4.07 (1H, ddd, J=3 x 3.37Hz, $H_{4'}$), 4.58 (1H, ddd, J=2 x 2.77, 5.69Hz, $H_{3'}$), 6.39 (1H, dd, J=6.08Hz, $H_{1'}$), 7.82 (1H, brs, H_2), 8.15 (1H, s, H_8); ^{13}C NMR (CD_3OD) δ 41.8 ($C_{2'}$), 62.5 (OCH_3), 63.5 ($C_{5'}$), 72.9 ($C_{3'}$), 86.7 ($C_{1'}$), 89.7 ($C_{4'}$), 93.1 (C_5), 95.1 (C_8), 120.2 (C_4), 139.5 (C_2), 147.1 (C_6); MS (EI) m/z (%) 135 (100, adenine$^{+\cdot}$), 165 (27.8, N^6-methoxyadenine$^{+\cdot}$), 192 (10.3), 205 (0.8), 221 (1.7), 236 (1.1, $M^{+\cdot}-NOCH_3$), 250 (1.2, $M^{+\cdot}-OCH_3$), 281 (1.3, $M^{+\cdot}$); (CI) m/z (%) 136 (47.2), 166 (6.9), 252 (54.9), 282 [6.2, $(M+H)^+$].

Nuclear Overhauser enhancement experiments: irradiation of the signal at 6.4ppm ($H_{1'}$) caused enhancement of signals due to H_8, $H_{4'}$, and $H_{2'b}$. Irradiation of the signal at 8.15ppm (H_8) caused enhancement of signals due to $H_{1'}$, $H_{3'a}$, and $H_{2'a}$.

2.4.2.7: Synthesis of N^6-methoxy-2′,3′-dideoxyadenosine (19)

N^6-Methoxyadenine (20mg, 0.12mmol) and 2′,3′-dideoxycytidine (77mg, 0.36mmol) were dissolved in citrate buffer (10ml, 10mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (1.0ml, 10%). The crude N-deoxyribosyltransferase extract (1ml, 15.8mg ml^{-1} protein, 7U) was added and the mixture was incubated for 4 weeks at 40°C. A further addition of enzyme was made every week but only minimal reaction was observed (<3% transfer). The progress of the reaction was followed by

reverse phase HPLC analysis (Techsphere 5C8 column, 25cm x 4.6mm, elution with 6% CH_3CN/10mM NH_4OAc). Due to the low transfer no new nucleoside was purified.

2.4.2.8: Synthesis of N^4-methoxy-5-methylcytidine (17)

O^4-Methoxyaminothymine (30mg, 0.19mmol) and 2′-deoxycytidine (132mg, 0.58mmol) were dissolved in citrate buffer (20ml, 10mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (2.0ml, 10%). The crude N-deoxyribosyltransferase extract (1ml, 16.3mg ml^{-1} protein, 8U) was added and the mixture was incubated for 2 weeks at 40°C. A further addition of enzyme was made after one week but no reaction was observed. The progress of the reaction was followed by reverse phase HPLC analysis (Techsphere 5C8 column, 25cm x 4.6mm, elution with 3% CH_3CN/10mM NH_4OAc).

2.4.2.9: Inhibition Studies of N^4-methoxy-5-methylcytosine (18)

N^4-methoxy-5-methylcytosine (1.0mmol) and crude N-deoxyribosyltransferase extract (0.1ml, 16.3mg ml^{-1} protein, ~1U) were dissolved in citrate buffer (0.3ml, 10mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (0.1ml, 10%). The mixture was incubated at 40°C and at intervals of 1, 3, and 6 h an aliquot (0.6ml) of the reaction mixture was removed and assayed for activity by the addition of 2′-deoxycytidine (3.0mmol) and adenine (1.0mmol) to a total volume of 1.0ml. The progress of the reaction was followed by reverse phase HPLC analysis (Techsphere 5C8 column, 25cm x 4.6mm, elution with 3% CH_3CN/10mM NH_4OAc).

2.4.2.10: Synthesis of 2′-deoxyguanosine (20)

2,6-Diaminopurine (20mg, 0.1mmol) and 2′-deoxycytidine (74mg, 0.3mmol) were dissolved in phosphate buffer (10ml, 1.0mM, pH 6.0). The crude N-deoxyribosyltransferase extract (200µl, 16.3mg ml^{-1} protein, 2U) was added and the mixture was incubated for 24 h at 40°C. The progress of the reaction was followed by reverse phase HPLC analysis (Techsphere 5C8 column, 25cm x 4.6mm, elution with 6% CH_3CN/10mM NH_4OAc). The reaction had reached >95% transfer to produce the 2,6-diaminopurine 2′-deoxynucleoside. Without purification the pH was adjusted to 7.4 by the addition of 1.0M NaOH and adenosine deaminase (10µl, 9.2mg ml^{-1} protein, 18U) was added to the mixture, which was incubated for 30 min at 25°C. The progress of the reaction was followed by reverse phase HPLC analysis (Techsphere 5C8 column, 25cm x 4.6mm, elution with 6% CH_3CN/10mM NH_4OAc). When the reaction had reached equilibrium the mixture was lyophilised. The residue was redissolved in distilled water (10ml, pH 5.0) and purified by ion exchange chromatography on Dowex 50 NH_4^+ (200-400 mesh). The required 2′-deoxyguanosine was eluted with distilled water (pH 5.0) to give 25mg (93% yield) of white crystals: UV (H_2O, pH 7.0) λ_{max} = 253.6nm; ^1H NMR (D_2O/NaOD) δ 2.33 (1H, ddd, J=4.45, 6.38. 13.6Hz, $H_{2'b}$), 2.62 (1H, ddd, J=2 x 6.70, 13.6Hz, $H_{2'a}$), 3.65 (1H, dd, J=~1, 5.03, 12.4Hz, $H_{5'a}$), 3.78 (1H, dd, J=~1, 3.31, 12.4Hz, $H_{5'b}$), 3.97 (1H, unresolved ddd, J=4.61Hz, $H_{4'}$), 4.46 (1H, ddd, J=2x4.29, 5.5Hz, $H_{3'}$), 6.22 (1H, dd appears as t, J=6.61Hz, $H_{1'}$), 7.83 (1H, d, J=0.99Hz, H_8); ^{13}C NMR (D_2O/NaOD) δ 40.6 ($C_{2'}$), 61.3 ($C_{5'}$), 72.1 ($C_{3'}$), 84.4 ($C_{1'}$), 89.1 ($C_{4'}$),118.5 (C_5), 136.6 (C_8), 151.52 (C_4), 161.66 (C_2), 168.81 (C_6); MS (FAB) m/z 267 ($M^{+\cdot}$). Nuclear Overhauser enhancement experiments: irradiation of the signal at 6.22ppm ($H_{1'}$) caused enhancement of signals due to H_8, $H_{4'}$, and $H_{2'b}$.

Irradiation of the signal at 7.83ppm (H_8) caused enhancement of signals due to $H_{1'}$, $H_{3'}$, and $H_{2'a}$.

2.4.2.11: Synthesis of 2′,3′-dideoxyguanosine (21)

2,6-Diaminopurine (10mg, 0.05mmol) and 2′,3′-dideoxycytidine (32mg, 0.15mmol) were dissolved in phosphate buffer (10ml, 1.0mM, pH 6.0). The crude N-deoxyribosyltransferase extract (1ml, 16.3mg ml^{-1} protein, 8U) was added and the mixture was incubated for 4 weeks at 40°C. A further addition of enzyme was made every week but only minimal reaction was observed (<5% transfer). The progress of the reaction was followed by reverse phase HPLC analysis (Techsphere 5C8 column, 25cm x 4.6mm, elution with 6% CH_3CN/10mM NH_4OAc). Due to the low transfer no new nucleoside was purified.

2.4.2.12: Synthesis of 9-β-D-2′-deoxyribofuranosyl 2-aminopurine (22)

2-aminopurine (20mg, 0.15mmol) and 2′-deoxycytidine (101mg, 0.45mmol) were dissolved in citrate buffer (10ml, 10mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (1.0ml, 10%). The crude N-deoxyribosyltransferase extract (0.5ml, 16.3mg ml^{-1} protein, 9U) was added and the mixture was incubated for 2 days at 40°C. The progress of the reaction was followed by reverse phase HPLC analysis (Techsphere 5C8 column, 25cm x 4.6mm, elution with 6% CH_3CN/10mM NH_4OAc). When the reaction had reached equilibrium, the mixture was lyophilised and the residue was purified by flash chromatography on silica gel (MeOH/CH_2Cl_2, 1/9) to give 30mg (80% yield) of white crystals: ^1H NMR (CD_3OD) δ 2.43 (1H, ddd, J=3.11, 6.20, 13.5Hz, $H_{2'b}$), 2.84 (1H, ddd, J=5.97, 7.67, 13.6Hz, $H_{2'a}$), 3.78 (1H, dd, J=3.87, 12.1Hz, $H_{5'a}$), 3.86 (1H, dd, J=3.44, 12.1Hz, $H_{5'b}$), 4.07

(1H, ddd, J=3 x 3.55Hz, $H_{4'}$), 4.62 (1H, ddd, J=2 x 2.98, 5.93Hz, $H_{3'}$), 6.42 (1H, dd, J=6.18, 7.62Hz, $H_{1'}$), 8.33 (1H, s, H_8), 8.60 (1H, s, H_6); ^{13}C NMR (CD_3OD) δ 40.98 ($C_{2'}$), 63.44 ($C_{5'}$), 72.85 ($C_{3'}$), 85.99 ($C_{1'}$), 89.45 ($C_{4'}$), 128.68 (C_5), 148.40 (C_8), 150.19 (C_6), 153.92 (C_4), 161.70 (C_2); HRMS (CI) m/z calcd ($C_{10}H_{12}N_5O_3$ + H^+) 252.1094, found 252.1096.

Nuclear Overhauser enhancement experiments: irradiation of the signal at 6.42ppm ($H_{1'}$) caused enhancement of signals due to H_8, $H_{4'}$, and $H_{2'b}$. Irradiation of the signal at 8.33ppm (H_8) caused enhancement of signals due to $H_{1'}$, $H_{3'}$, and $H_{2'a}$. Irradiation of the signal at 8.60ppm (H_6) caused no enhancements of any signals.

2.4.2.13: Synthesis of 9-β-D-2′,3′-dideoxyribofuranosyl 2-aminopurine (23)

2-Aminopurine (20mg, 0.15mmol) and 2′,3′-dideoxycytidine (94mg, 0.45mmol) were dissolved in citrate buffer (50ml, 10mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (5.5ml, 10%). The crude N-deoxyribosyltransferase extract (0.5ml, 16.3mg ml^{-1} protein, 9U) was added and the mixture was incubated for 12 days at 40°C. The progress of the reaction was followed by reverse phase HPLC analysis (Techsphere 5C8 column, 25cm x 4.6mm, elution with 6% CH_3CN/10mM NH_4OAc). When the reaction had reached equilibrium, the mixture was lyophilised and the residue was purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 3/97) to give 20mg (57% yield) of white crystals: ^1H NMR (CD_3OD) δ 2.20 (2H, m, $H_{3'a \text{ and } b}$), 2.55 (2H, m, J=5.97, 7.67, 13.6Hz, $H_{2'a \text{ and } b}$), 3.70 (1H, dd, J=4.30, 12.1Hz, $H_{5'a}$), 3.87 (1H, dd, J=3.23, 12.1Hz, $H_{5'b}$), 4.28 (1H, dddd appears as septet, J=2 x 3.79, 2 x 7.41Hz, $H_{4'}$), 6.28 (1H, dd appears as t, J=2 x 5.22Hz, $H_{1'}$), 8.40 (1H, s, H_8), 8.59 (1H, s, H_6); ^{13}C NMR (CD_3OD) δ 26.62 ($C_{3'}$), 33.20 ($C_{2'}$), 64.30 ($C_{5'}$), 64.50 ($C_{4'}$), 83.48 ($C_{1'}$), 128.60 (C_5), 143.22

(C_8), 149.88 (C_6), 153.85 (C_4), 161.78 (C_2); HRMS (CI) m/z calcd ($C_{10}H_{12}N_5O_2$ + H^+) 236.1145, found 236.1147.

Nuclear Overhauser enhancement experiments: irradiation of the signal at 6.28ppm ($H_{1'}$) caused enhancement of signals due to H_8, $H_{4'}$, $H_{3'b}$, and $H_{2'b}$. Irradiation of the signal at 8.40ppm (H_8) caused enhancement of signals due to $H_{1'}$, $H_{3'a}$, and $H_{2'a}$. Irradiation of the signal at 8.60ppm (H_6) caused no enhancements of any signals.

2.4.2.14: Synthesis of 2-Thio-2'-deoxyuridine (24)

2-Thiouracil(15mg, 0.11mmol) and thymidine (80mg, 0.35mmol) were dissolved in citrate buffer (10ml, 1.0mM, pH 6.0 containing 0.05% sodium azide) and ethylene glycol (1.0ml, 10%). The crude N-deoxyribosyltransferase extract (1.0ml, 16.3mg ml^{-1} protein, 18U) was added and the mixture was incubated for 1 day at 40°C. The progress of the reaction was followed by reverse phase HPLC analysis (Techsphere 5C8 column, 25cm x 4.6mm, elution with 2% CH_3CN/10mM NH_4OAc). When the reaction had reached equilibrium, the mixture was lyophilised and the residue was purified by flash chromatography on silica gel (MeOH/CH_2Cl_2, 1/18) to give 14mg (47% yield) of a white crystalline solid: Rf = 0.17 (CH_2Cl_2/MeOH, 9/1): ^1H NMR (CD_3OD) δ 2.20 (1H, ddd appears as sextet, J=12.8, 9,03, 6.14Hz, $H_{2'a}$), 2.57 (1H, ddd appears as dt, J=6.03, 5.08Hz, $H_{2'b}$), 3.68 (1H, dd, J=4.42, 12.6Hz, $H_{5'b}$), 3.79 (1H, dd, J=3.07, 12.8Hz, $H_{5'a}$), 4.02 (1H, ddd appears as q, J=3 x 3.91Hz, $H_{4'}$), 4.29 (1H, ddd appears as q, J=3 x 4.54Hz, $H_{3'}$), 6.35 (1H, dd, J=6.02, 8.99Hz, $H_{1'}$), 6.66 (1H, d, J=7.6Hz, H_5), 8.16 (1H, d, J=7.6Hz, H_6); MS (CI) m/z 128 (76), 243 [9, ($M+H^+$)].

Nuclear Overhauser enhancement experiments: irradiation of the signal at 6.35ppm ($H_{1'}$) caused enhancement of signals due to H_8, $H_{4'}$, and $H_{2'b}$.

Irradiation of the signal at 8.16ppm (H_6) caused enhancement of signals due to $H_{1'}$, $H_{3'}$, and $H_{2'a}$.

CHAPTER 3

IMMOBILISED ENZYMES

3.1: Background

Chemists have been aware for a long time of the advantages that the use of enzymes as catalysts have over purely chemical means. Enzymes demand attention chiefly because of the efficiency and selectivity with which they catalyse reactions and the mildness of their reaction conditions but their use has been restricted because their stability is often limited. This problem can usually be overcome by immobilising the enzyme on a solid support and thus enhancing its thermal, mechanical and chemical stability to produce a recoverable catalyst. The ease of separation of the immobilised enzyme from the reaction mixture, its extended life-span and the possibility of reuse all offer considerable operational advantages over the corresponding free enzyme. The physical and physicochemical properties of immobilised enzymes have been shown, in some cases, to differ from those of their counterparts in free solution, such as pH-activity, kinetic parameters, substrate specificity and stability. These immobilised enzymes have found many uses in industrial, analytical and medical procedures[82, 167-172].

An immobilised enzyme molecule is prevented from diffusing freely through the reaction medium by being attached physically and/or chemically to a support material. Chemical methods are usually irreversible whereas physical techniques, such as hydrophobic interactions, should, in theory, be completely reversible. The reaction system thus consists of two phases: the bulk solution, and the immobilised

enzyme with its support environment. The support materials are usually insoluble in water and are often high molecular weight, hydrophilic polymers. There are four general methods for immobilising enzymes[82]: 1) covalent binding, 2) adsorption, 3) entrapment and 4) encapsulation.

3.1.1: Covalent Binding

Immobilisation by covalent bonding of an enzyme to an activated polymer is probably the most extensively used method. The outer surface of the enzyme contains many different amino acid functional groups which are suitable for participation in the covalent bond formation. As the support matrix is more robust than the enzyme, this is activated before addition of the enzyme. The activated functional groups on the support then react with the ones present on the enzyme surface, under much milder conditions. A number of amino acid functional groups which are most often involved in the covalent binding are: the amino group (NH_2) of lysine or arginine; the carboxyl group (CO_2H) of aspartic acid or glutamic acid; the hydroxyl group (OH) of serine or threonine; and the sulphydryl group (SH) of cysteine.

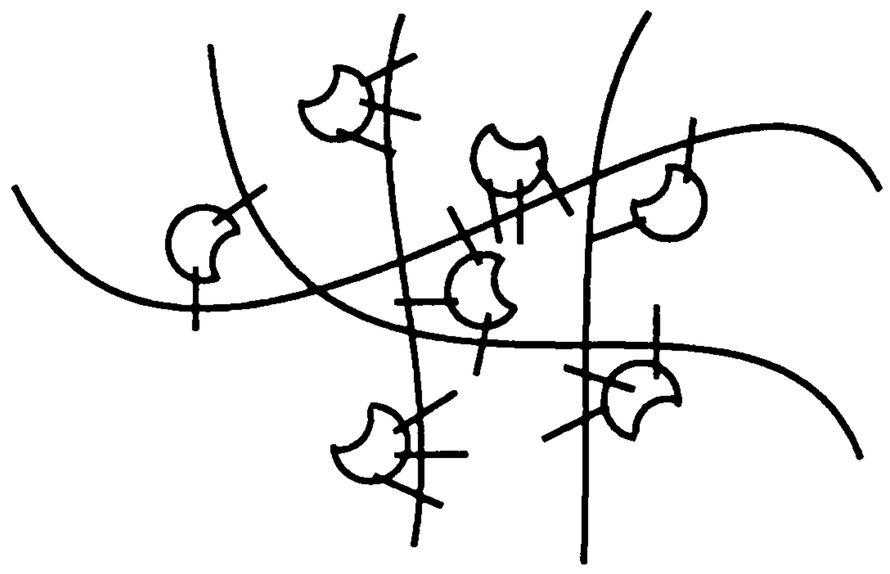


Fig. 3.1 Immobilisation by covalent binding

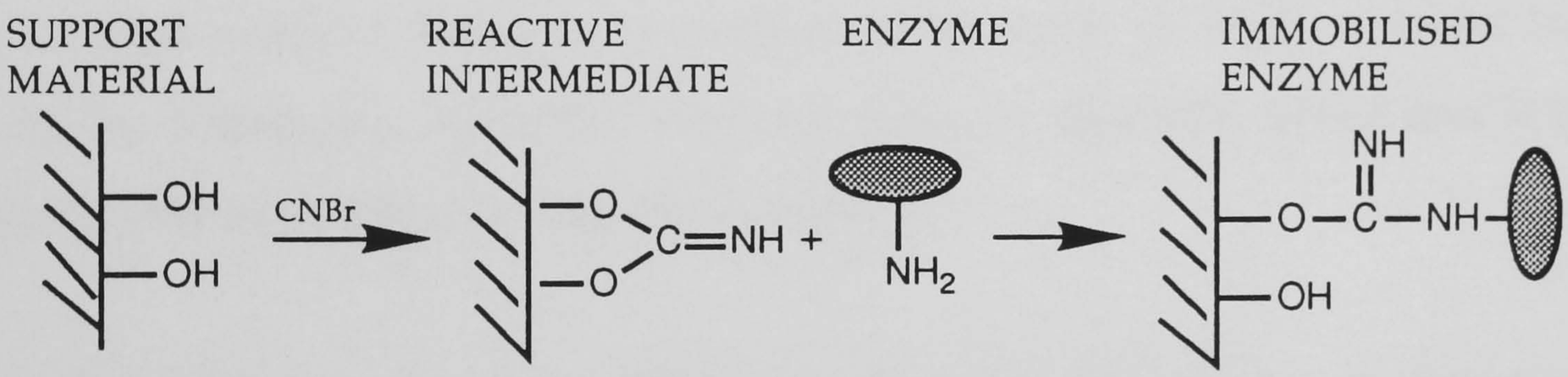
There are many support materials available for covalent binding and the advantages and disadvantages of each must be considered for each required application. Hydrophilicity is the most important factor for maintaining enzyme activity in a matrix environment. Therefore, biological polysaccharide polymers, which are very hydrophilic, such as cellulose, dextran (Sephadex), starch and agarose (Sepharose) are widely used for enzyme immobilisations. Sepharose and Sephadex are the two most commonly used supports; they are spherical bead forms of the polymeric natural products and possess superior mechanical strength compared with the fibrous polysaccharide, are of defined physical dimensions and have known pore sizes. The hydroxyl groups on the sugar residues in these polymers are ideal for covalent bonding and can also hydrogen bond with water to form micro-cavities and small channels. These beads are very strong and durable but are less hydrophilic than the polysaccharide materials, such as cellulose. Organic supports such as polystyrene and polyacrylamide can also covalently bond to the enzyme and form cross-linked networks of fibres which form a hydrophilic environment.

There are many reactions for joining an enzyme to the immobilisation matrix by a covalent bond such as:

- formation of an isourea linkage;
- formation of a diazo linkage;
- formation of a peptide bond;
- an alkylation reaction.

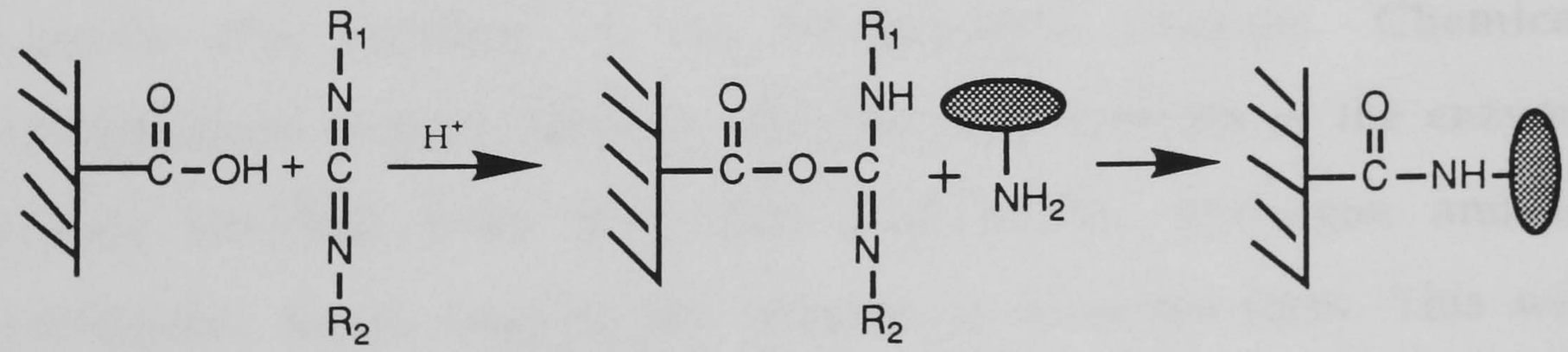
The favoured method is chosen so as not to react with any groups which may be important in the catalytic action of the active site of the enzyme. The residues at the active site of the enzyme must be avoided when coupling takes place, and their protection can be achieved by the use of substrate or reversible inhibitors. Similarly, coupling should not take place through residues involved in allosteric control or subunit-subunit interactions.

The functional groups on the support material are activated first by a specific reagent to make them strongly electrophilic. The enzyme is then added and the amino acid functional groups nucleophilically attack the activated groups to form the covalent bond.



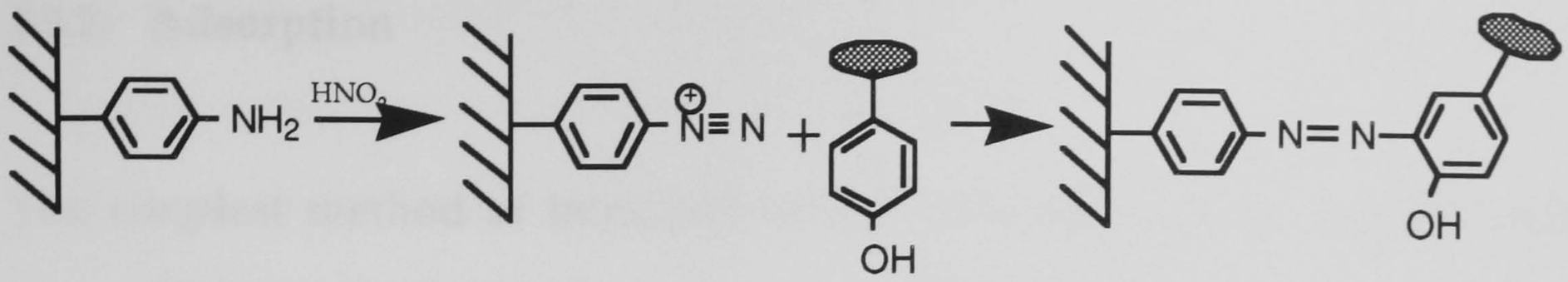
Scheme 3.2 Cyanogen bromide activation

Cyanogen bromide is used to activate the hydroxyl groups of polysaccharide support materials.



Scheme 3.3 Carbodiimide activation

Carbodiimide activation involves the bonding of the carboxyl group and the enzyme via a peptide bond.



Scheme 3.4 Nitrous acid activation

Aromatic amine groups on the support can be diazotised using nitrous acid. The bond is formed between the reactive diazo group of para-aminobenzoyl on the support and the ring structure of an aromatic amino acid such as tyrosine.

However, hydroxyl ions from the aqueous solvent can also react with the activated support therefore, activation levels must be high to allow for loss by hydrolysis. Activated materials must be carefully stored and it is preferably to use them as quickly as possible.

Normal functional groups on a support can be chemically modified to produce a range of derivatives containing different functional groups. For example, the hydroxyl group in cellulose can be modified to produce CM-cellulose (carboxymethyl), and DEAE-cellulose (diethylaminoethyl). Derivatization can also modify the charges on the surface of a support to improve the binding of the immobilised enzyme. Chemical immobilisation is more likely to alter the characteristics of the enzyme because covalent links may alter electrostatic, hydrogen and/or hydrophobic bonds keeping the enzyme in its active form. This will therefore affect the catalytic ability, stability and activity of the immobilised enzyme. Loss of activity may arise in a multi-unit enzyme if dissociation of the subunits occurs to leave only one of the subunits covalently attached to the support.

3.1.2: Adsorption

The simplest method of immobilisation is adsorption of an enzyme onto the support material. However, the ease with which binding is achieved is reflected in the ease with which the enzyme may also be removed. No pre-activation steps are required to alter the functional groups on the support as the method relies on the adhesion of the enzyme to the support material by non-covalent interactions, such as ionic interactions, van der Waal's forces, hydrophobic effects and hydrogen bonding.

The outer surface of the enzyme may have regions which are hydrophobic, hydrophilic, charged or neutral and these properties will vary in distribution and dominance with the surrounding conditions of pH, molarity, hydrophilicity and temperature. Support materials with complementary features will adsorb the biocatalyst with a strength and specificity related to the extent of the complementarity. The strength of these interactions can be increased by the introduction of strong, charged groups, as in ion exchange resins; or by the incorporation of an ion which forms strong complexes with proteins, such as titanium (IV) ions; or by the use of a specific affinity ligand such as an antibody to the biocatalyst. There are many support materials available, such as alumina, activated carbon, kaolinite, bentonite, porous glass, anion-exchange resins (e.g. DEAE-Sephadex) and cation-exchange resins (e.g. CM-cellulose).

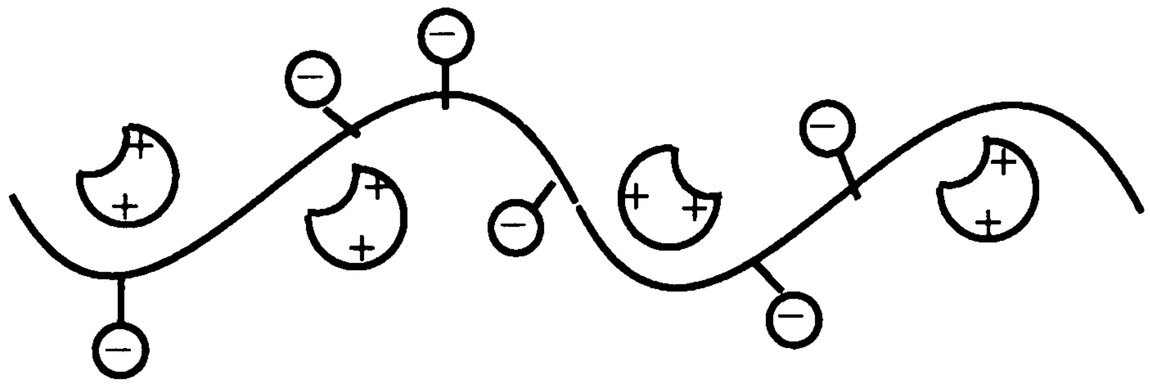


Fig. 3.5 Immobilisation by adsorption

The enzyme is bound to the support by mixing the aqueous solution of both for a period of time, after which any excess enzyme is washed away. Problems with adsorption of other ions and unwanted proteins which compete for the adsorption sites are common place. The pH and ionic strength must be carefully monitored as any fluctuations can alter the charges on both the enzyme and the support, resulting in the release of the enzyme from the support. Although leakage of the enzyme occurs due to the weak non-covalent bonds, this method is cheap, simple and the support can be regenerated with fresh enzyme. As with covalent immobilisation, the properties of the enzyme can also be altered by adsorption, due to the great influence of ionic or steric repulsion of the support material on enzyme activity.

3.1.3: Entrapment and Encapsulation

The immobilisation of enzymes by entrapment or encapsulation differs from the other methods discussed, in that the enzyme molecules are free in solution, but restricted in movement by the three-dimentional lattice of a gel or by a semi-permeable membrane. There is minimum effect on the enzyme since the enzyme is still in solution but within a defined physical volume.

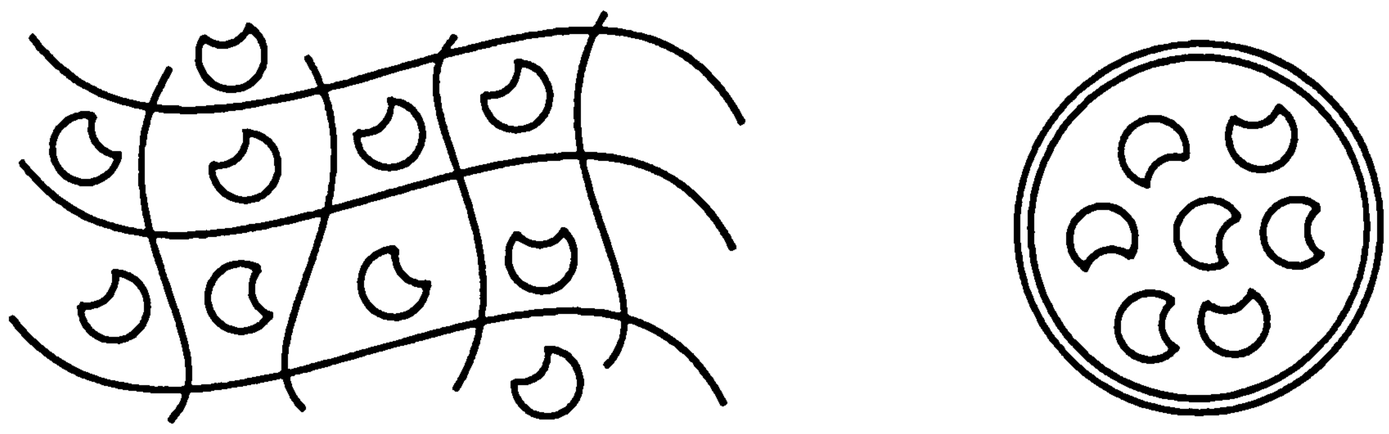


Fig. 3.6 Immobilisation by entrapment and encapsulation

The porosity of the gel lattice or semi-permeable membrane is controlled to ensure that the structure is tight enough to prevent leakage of the enzyme but at the same time allow free diffusion of substrate and product molecules.

3.1.3.1: Entrapment

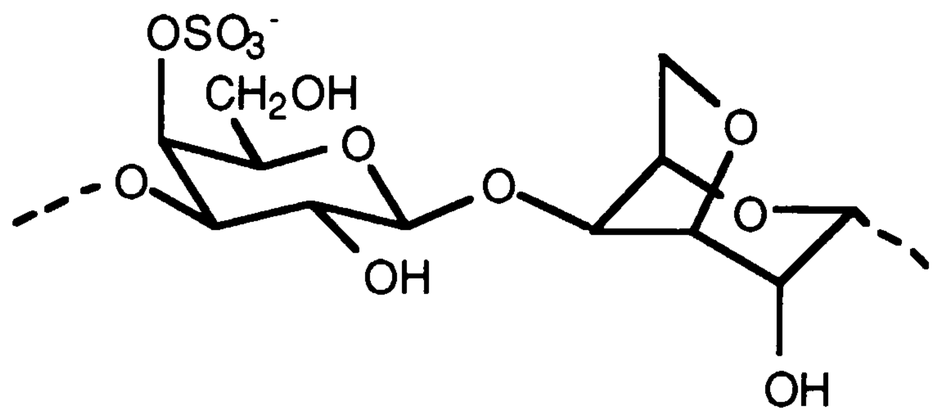
Entrapment can be achieved by mixing the enzyme with a fluid precursor of the gel and subsequently inducing gelation, either by polymerisation or precipitation. For example, polymerisation of acrylamide monomers to form polyacrylamide or methyl acrylate to form polymethacrylate. Cross-linking agents can also be added during polymerization to create a three-dimentional lattice. The relative amounts of monomer and cross-linking agent can be varied to determine the pore size and mechanical properties of the gel.

Alginic acid cross-linked with calcium ions has proved useful for cell entrapment but often the gel is too open to retain enzymes and calcium chelation can eventually lead to loss of gel structure. Some gelling materials, such as carrageenan, are sold in grades specifically for immobilisation work.

3.1.3.2: k-Carrageenan

Carrageenans are sulphated derivatives of polysaccharides obtained from some seaweeds, and are often used as a food additive. There are three principal types of natural carrageenan κ,ι,and λ. Carrageenans are mainly composed of D-galactose, 3,6-anhydro-α-D-galactose and about 20-30% of sulphate groups to produce a molecular weight of 100,000 to 800,000[167]. The

characteristics of κ-carrageenan, compared to the other types, make it the more suitable for immobilisation purposes[173, 174].



D- glactase-4-sulphate 3,6-anhydro-D-galactose

Fig. 3.7 Limiting structure of κ-carrageenan

The bonding of the carbohydrate rings leads to the formation of a double helix. κ-Carrageenan is easily induced to gel by contact with metal ions, e.g. K^+,Rb^+, and Cs^+ ions, amines, e.g. hexamethylenediamine, amino-acid derivatives, e.g. DL-histidine hydrazine, water-miscible organic solvents and cooling below 10°C. Inclusion of cells or enzyme in the fluid prior to gelling results in a mild and efficient immobilisation method. The preparations can be formed into cubes, beads or membranes and used in various forms of continuous or batch reactors.

3.1.3.3: Encapsulation

Encapsulation of enzymes can be achieved by enveloping the molecules within various forms of semi-permeable membranes. Once again, the enzyme is prevented from passing out of the immobilisation medium but small substrate and product molecules can pass freely across the membrane. Microcapsules varying from 10μl to 1000μl diameter have been made from many materials such as nylon and cellulose nitrite. Biological cells, such as erythrocytes can be used and liposomes can be formed from

lipids to produce two-layered structures similar to that found in cell membranes.

Both these methods have the disadvantage that leakage of enzyme can be high and often only low yields of immobilisation are achieved. These methods have a more limited use than the other techniques. However, by combining entrapment with covalent bonding a very useful technique has been developed[175, 176].

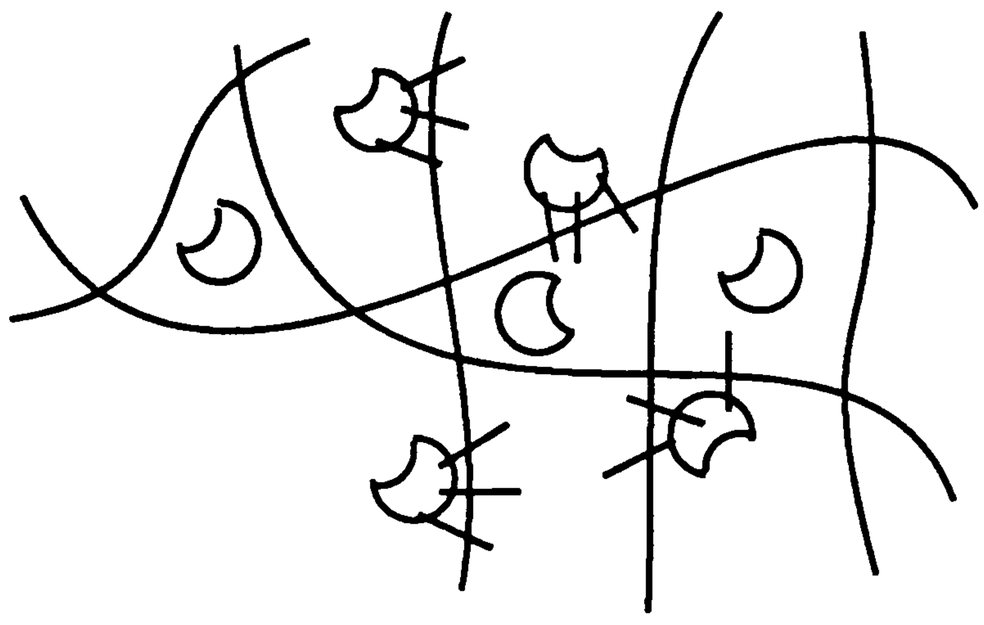


Fig. 3.8 Immobilisation by combined covalent bonding and matrix entrapment

The procedure involves the simultaneous reaction of three components in neutral buffered aqueous solution at room temperature: a copolymer of acrylamide and N-acryloxysuccinimide, a low molecular weight α,ω-diamine, and the enzyme. The copolymer, poly(acrylamide-co-N-acryloxysuccinimide) (PAN) is prepared by the azobis(isobutyronitrile) (AIBN) catalysed reaction of acrylamide and N-acryloxysuccinimide. The water-soluble copolymer is then cross-linked by reaction of the α,ω-diamines with the active ester groups of the PAN to form an insoluble gel connected through amide groups. The addition of enzymes to the reaction, as cross-linking takes place, results in reaction of the lysine ε-amino functions of the enzyme to the gel through additional amide linkages. In

the presence of saturating concentrations of reagents which bind at the active site, the enzyme is maintained in its active conformation during the covalent bond formation and the active site residues are protected from the covalent bonding.

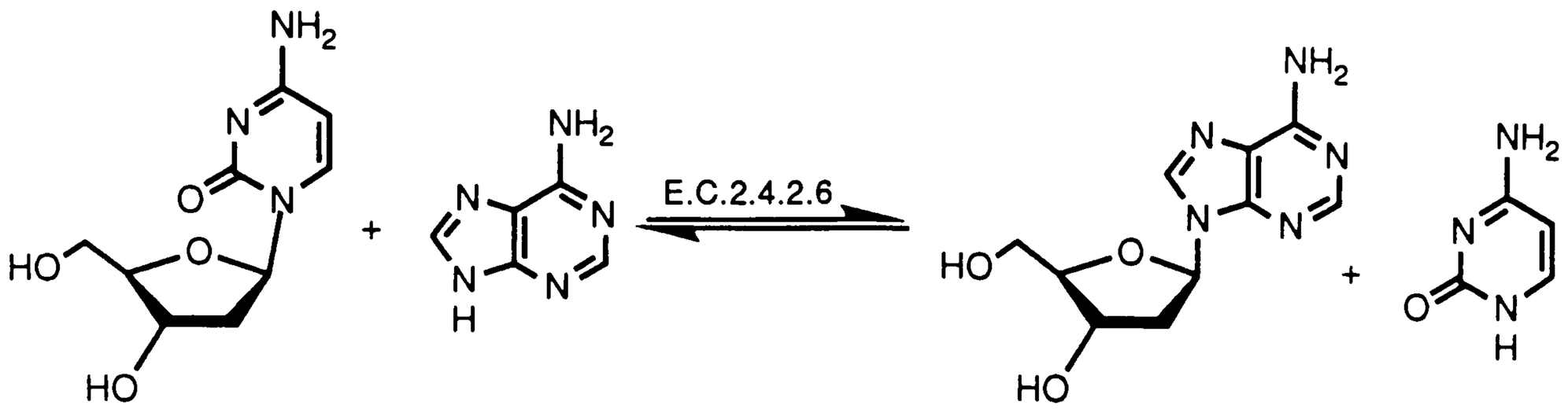
3.1.4: Applications of Immobilised Enzymes

Due to the vast array of techniques and immobilisation supports available, it is necessary to choose a particular method to meet the specific requirements of any investigation. In this project the use of immobilised N-deoxyribosyltransferase from *Lactobacillus leichmannii* was investigated for the batch production of nucleoside analogues. In the large scale production, enzyme immobilised on a suitable support can be rapidly removed from reaction mixtures and have the advantage of being reusable. In order to be able to select a suitable immobilisation method, several supports must be investigated to evaluate their usefulness in retaining and stabilising the enzyme for repeated use.

3.2: RESULTS AND DISCUSSION

3.2.1: Standard Assay of Immobilised Nucleoside Deoxyribosyltransferase

Three distinctly different methods of enzyme immobilisation were investigated to find which method and matrix support would give the most stable enzyme preparation for use in a batch reactor. Each method was assay by the standard glycosyl transfer reaction:



dC A dA C

Scheme 3.9 Standard assay for immobilised enzyme activity

As all the nucleosides in this reaction absorb in the UV, the reaction was followed by reverse-phase HPLC using a UV detector at λ=254nm (as described in Chapter 2). The assay method for each immobilisation support is described in the Experimental section.

3.2.2: Immobilisation on Hydrophobic Supports

The hydrophobic nature of the proteins and enzymes in the crude preparation were investigated by trying to immobilise the crude enzyme preparation by adsorption. Three different columns were prepared containing agarose with different groups covalently attached (phenyl, trityl, and octyl). An equal amount of enzyme was applied to each column and allowed to equilibrate at room temperature. Each column was washed with buffer and the eluate collected for analysis of the unbound activity. These initial washings showed a small amount of enzyme activity but most was retained on the column. The immobilised enzyme was assayed every hour to measure the retention of activity on the column.

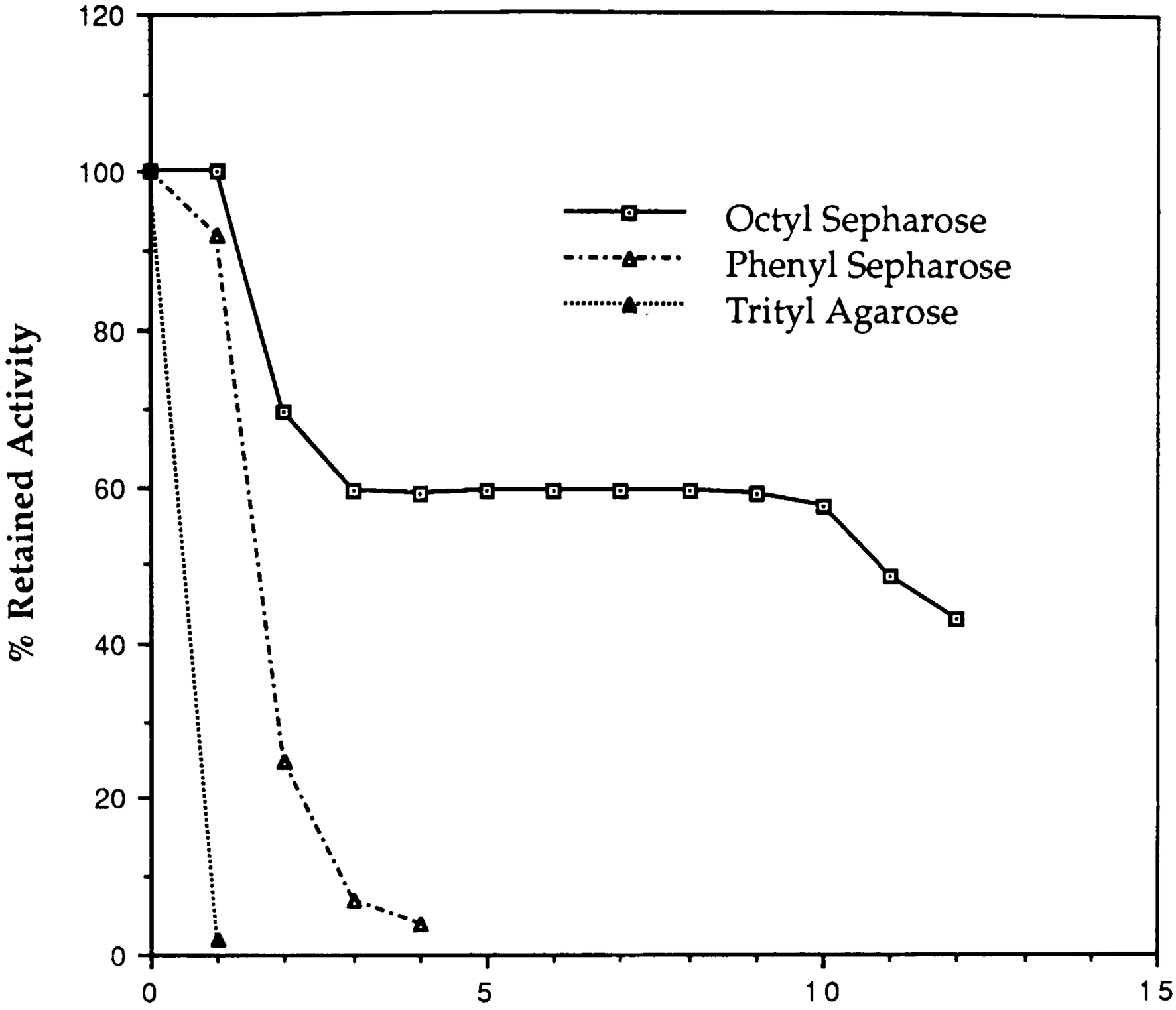


Fig. 3.10 Retention of N-deoxyribosyltransferase on hydrophobic Sepharose

3.2.2.1: Octyl Sepharose

As can be seen on the graph, the most promising results were found with octyl Sepharose. When the enzyme was immobilised on the octyl Sepharose as described in the Experimental, considerable activity was retained on the column. The initial eluate was collected for analysis and negligible activity was found. The immobilised enzyme was subjected to numerous assay cycles and it was found that good levels of activity (59%) remained after ten cycles. After this the levels of activity started to decrease

slowly. The levels of retained activity could have been affected by several factors: initially losses in activity might have be due to the elution of the active enzyme from the octyl Sepharose; later on after the percentage of retained activity had levelled out, any losses in activity may have been due to deactivation of the N-deoxyribosyltransferase retained on the column. After the first few washings there was no enzyme activity measured in the eluates so therefore, it was assumed that the losses in activity were due to deactivation. This deactivation may be caused by the dissociation of the subunits of this hexameric enzyme[116].

3.2.2.2: Immobilised Enzyme Stability at 4°C

A sample of the octyl Sepharose immobilised N-deoxyribosyltransferase was stored at 4°C and assayed every day to ascertain the levels of retained activity. Similar levels of activity to the continuous assay described above were obtained. However, after seven cycles the levels activity started to decrease due to deactivation of the immobilised enzyme. It was decided that the simplicity of immobilisation on octyl Sepharose was conducive to the preparation of the immobilised enzyme when it was required and there was no need to prepare and store the enzyme in this form.

3.2.2.3: Phenyl and Trityl Sepharose

The adsorption of N-deoxyribosyltransferase onto phenyl and trityl Sepharose was performed as described for octyl Sepharose. However, unlike octyl Sepharose, the retention of activity on the column was very poor. The initial washings of both columns showed a small amount of enzyme activity but most was retained on the column. After only a few assay cycles the levels of activity on both columns was very low and when

the eluted fractions were assayed, by a Bio-Rad assay (as described in Chapter 2), they showed the presence of enzyme. The loss in activity in this case was due to elution of the enzyme and not due to any deactivation.

As a result of these findings, further investigation into immobilisation on phenyl or trityl Sepharose was abandoned. The retained activity found on the octyl Sepharose column have a reasonable life-span but before any conclusions as to the preferred method of immobilisation were made, other methods were investigated.

3.2.3: Immobilisation by Entrapment using κ-Carrageenan

κ-Carrageenan was chosen as the support matrix to investigate for the entrapment of N-deoxyribosyltransferase. The solid cubes of the immobilised enzyme were prepared as described in the Experimental and placed in a filtration unit to make a batch reactor. The cubes were washed with citrate buffer and the eluate collected for analysis of the unbound activity. These initial washings showed a small amount of enzyme activity but most was retained in the κ-carrageenan cubes. The immobilised enzyme was assayed every two hours to measure the retention of activity in the cubes.

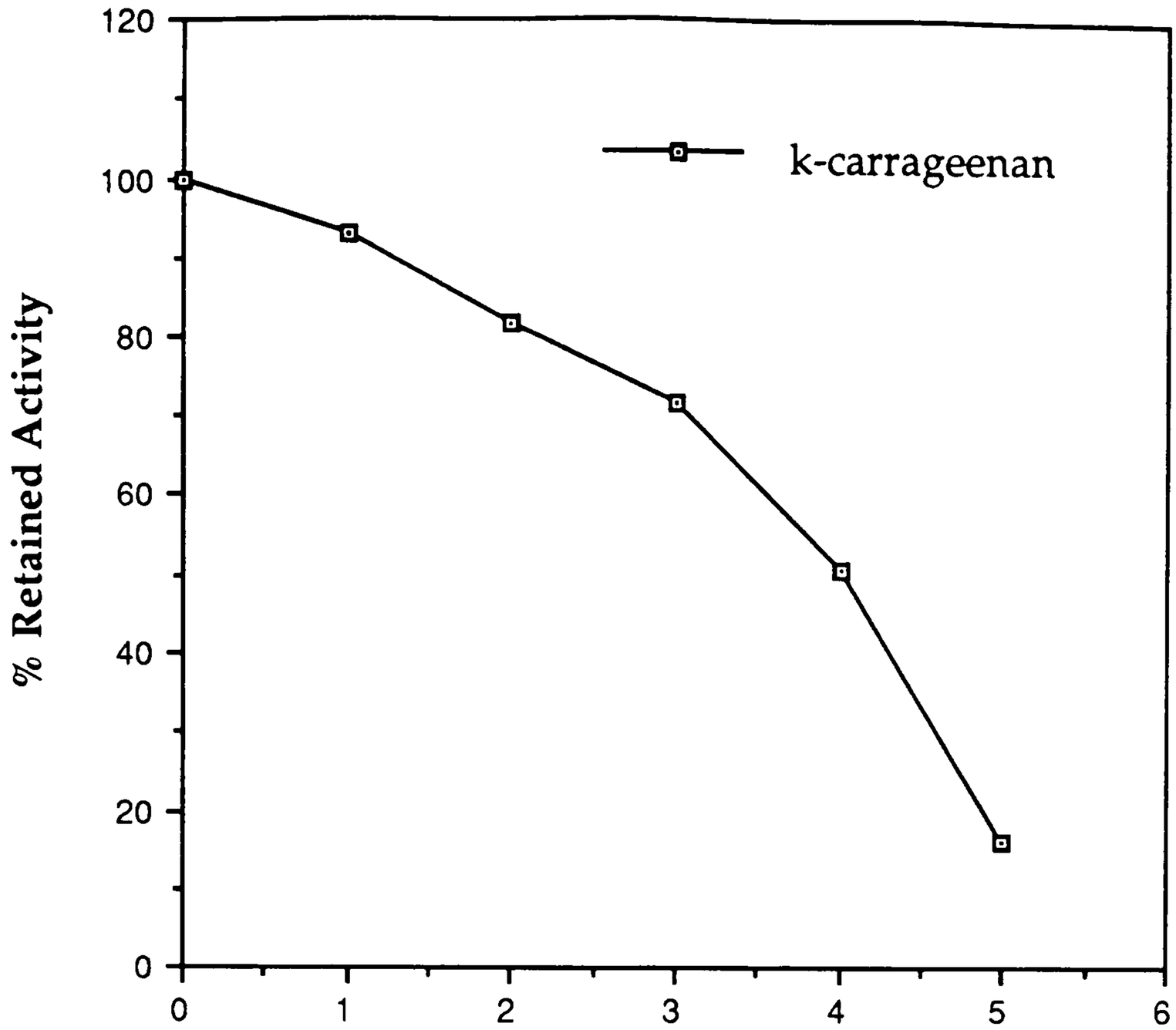


Fig. 3.11 Retention of N-deoxyribosyltransferase by entrapment in κ-carrageenan

Considerable activity appeared to be retained in the κ-carrageenan. The immobilised enzyme was subjected to numerous assay cycles but it was found that the levels of activity fell after each assay cycle due to leakage of the enzyme from the κ-carrageenan. The presence of enzyme in the eluted assay mixtures was confirmed by a Bio-Rad protein assay (as described in Chapter 2).

To try to prevent enzyme leakage from the κ-carrageenan cubes, different concentrations of KCl solution were investigated in the hardening of the gel but this was found to have very little effect. During the assay cycles the κ-carrageenan gel remained reasonably intact in the cubic form in which it

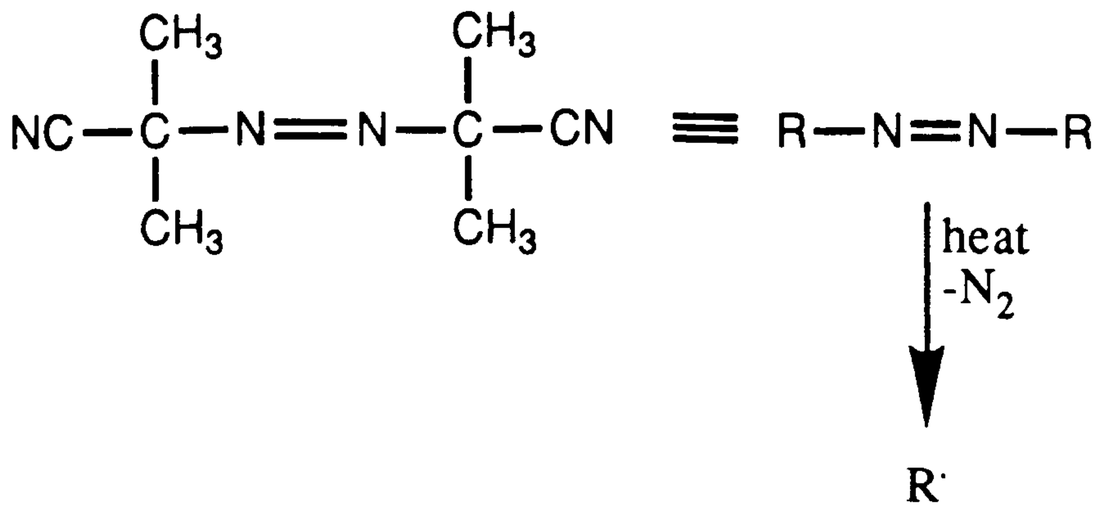
had been moulded so it was felt that enzyme leakage was partly due to the collapse of the gel structure, but was more likely to be related to the size of the pores in the gel structure which were obviously too large to retain the enzyme. The pores not only allowed diffusion of the substrate molecules in and out of the gel matrix but also diffusion of the enzyme was possible. As an obvious continuation from these results the use of a combined method of entrapment and covalent binding was investigated.

3.2.4: Covalent Binding and Entrapment

Due to the problems encountered in the adsorption and entrapment of the enzyme, a combination of covalent binding and entrapment was investigated. The method of Pollak *et al.*[175] was used to synthesise the polymer and to immobilise the enzyme as described in the Experimental. This method was reported to be applicable to small quantities of enzyme, to give high yields of immobilised activity and to incorporate a link between the enzyme and the immobilising matrix which is hydrolytically stable. The enzyme is incorporated into a hydrophilic medium and the matrix produced has a high permeability towards low molecular weight substrates.

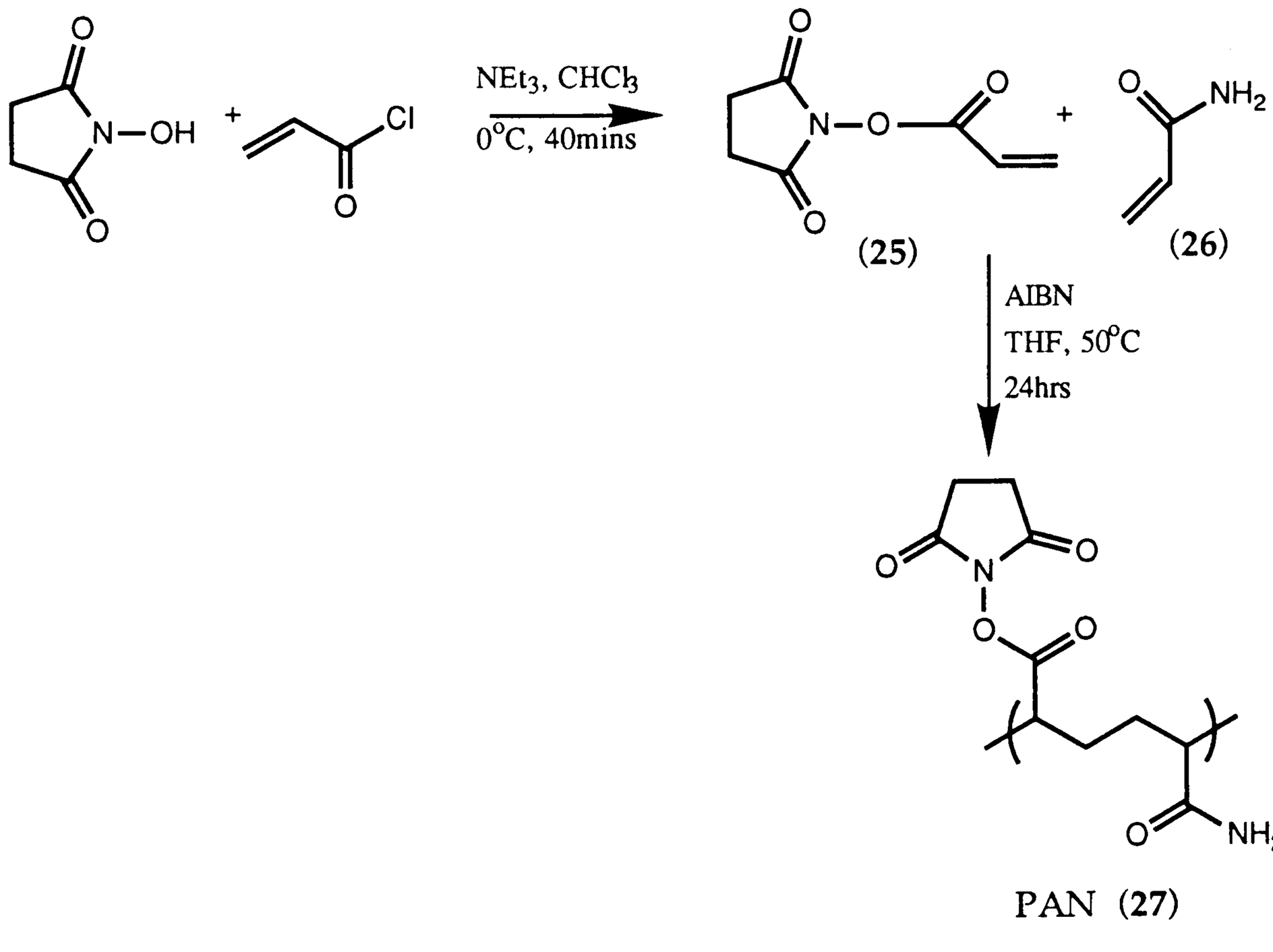
3.2.4.1: Preparation and Characterisation of PAN

PAN (**27**) was prepared by the free-radical polymerisation of acrylamide (**25**) and N-acryloxysuccinimide (**26**) in THF solution, using thermal initiation with azobis(isobutyronitrile) (AIBN).



Scheme 3.12 The thermal decomposition of the radical initiator AIBN

This method produced low molecular weight polymers as a white solid. The polymer was very stable at room temperature in a desiccator under dry air but the active ester groups hydrolyzed very quickly in the presence of moisture.



Scheme 3.13 Synthesis of PAN

3.2.4.2: Assay for the Active Ester Content of PAN

The polymer was assayed for active ester groups by allowing it to react with aqueous propylamine solution and measuring the absorbance due to the anion of N-hydroxysuccinimide. The rate of appearance of N-hydroxysuccinimide was followed spectrophotometrically at λ=259nm at room temperature. After the reaction was completed (~60 min) the active ester concentration was calculated. The absorbance due to the anion of N-hydroxysuccinimide is equal to the final absorbance minus the initial absorbance (ε=8600$M^{-1}cm^{-1}$). Therefore, using Beer-Lambert law, the concentration of active ester groups was found to be 573µmol per gram in this sample. The composition of the polymer was specified in the paper in terms of the content (in µequiv/g) of active ester groups: thus, PAN-550 is a polymer which releases 550 (±25) µmol of N-hydroxysuccinimide per gram of dry polymer on treatment with excess aqueous propylamine solution. To avoid confusion resulting from unnecessary precision, these descriptive numbers of active ester groups are rounded to the nearest 50; thus a polymer assayed to contain 573µequiv/g of active ester groups is called PAN-550.

3.2.4.3: Determination of the Gel Time

The rate of gel formation can be altered by changing the temperature or pH of the coupling reaction. Before performing an immobilisation using PAN it was necessary to investigate the time the gel would take to form and to adjust this as necessary, as gels taking longer than 5 min to form are soft because of the hydrolysis of the active ester groups. To increase the rate of the coupling reaction the temperature can be raised or the pH can be made more basic; by reversing these conditions the gel time can be lengthened.

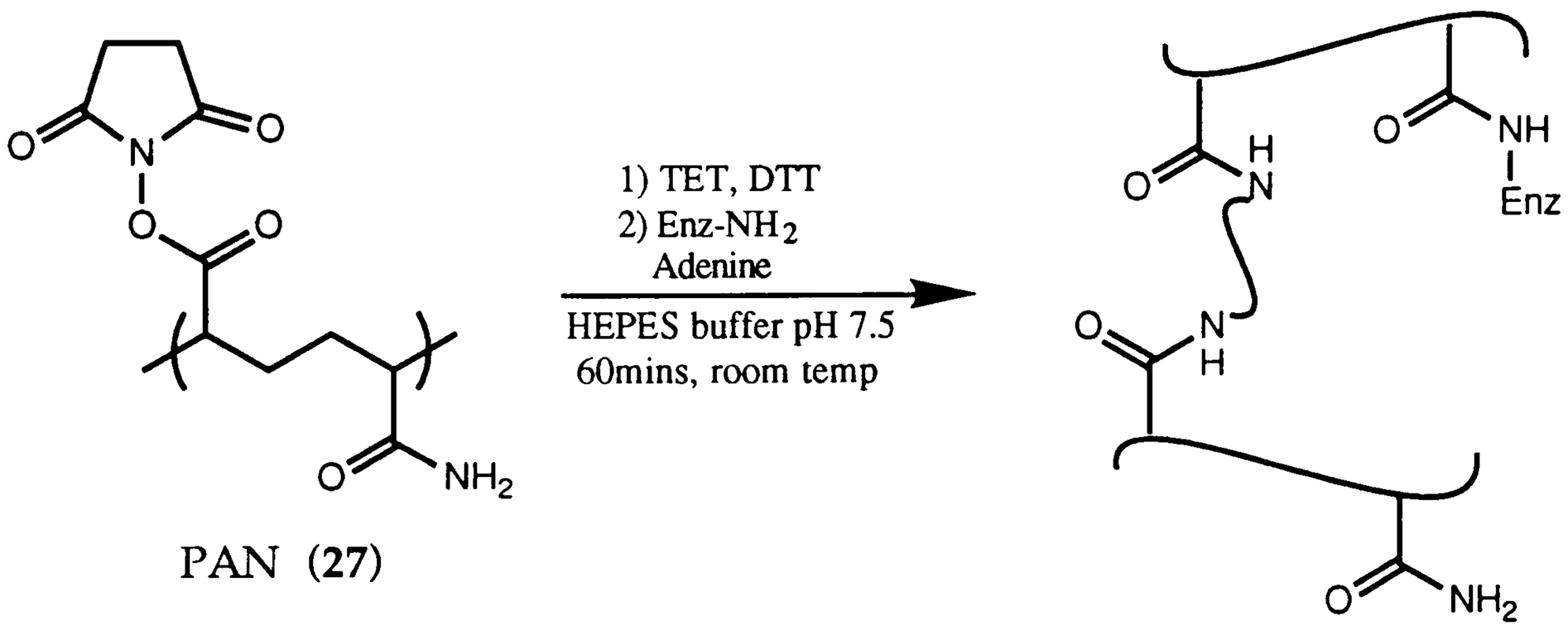
To produce a gel which has the correct mechanical properties for enzyme immobilisation requires the following composition: the reaction of a 20% solution of PAN (0.3M Hepes buffer, pH 7.5) having 450-1550 μequiv of active ester groups/g with a quantity of TET providing 0.85 equiv of primary amine groups/equiv of active ester. This stoichiometry leaves ~15% of the active ester groups unreacted and available for reaction with the nucleophilic groups on the enzyme. Therefore, addition of the N-deoxyribosyltransferase to the reacting solution of PAN and TET before gelling is complete will result in its covalent incorporation into the cross-linked polymer network.

The corresponding quantities of each reagent, as outlined above, were mixed together without the addition of the enzyme to ascertain the gel time. After 2 min the mixture had set to a transparent, mechanically resilient gel. Therefore, this method seemed to be suitable for the immobilisation of the enzyme.

3.2.4.4: Immobilisation of N-Deoxyribosyltransferase on PAN-550

In the standard procedure, a quantity of PAN sufficient to give a 20% w/w solution was weighed rapidly in air and dissolved in 0.3M Hepes buffer containing components intented to protect enzymatic activity. In the case of N-deoxyribosytransferase, a nucleoside or base was added to occupy the active site. The substrate should bind to the active site of the enzyme and prevent modification of nucleophiles close to or at the active site by acylation with PAN during the immobilisation. 1,4-Dithiothreitol (DTT), a reducing thiol, was also added during the immobilisation to inhibit protein autoxidation for enzymes containing essential catalytic or structural thiol groups. As the structure of N-deoxyribosyltransferase has

not been published it was assumed that the enzyme might contain some stucturally important disulphide bridges, although it is known that there are no essential catalytic thiol groups in the active site[114]. The cross-linking agent, TET was added at the same time as DTT and the gelling process started. About 30 sec after the addition of TET, the enzyme solution (10mg/g of PAN) was added to the reaction mixture. After preliminary investigations it was found that the highest yields of immobilised activity were obtained using quantities of enzyme not greater than 10mg/g of PAN. For high yields of immobilised activity, the enzyme must be added after about one-third of the gel time so that there is enough time for thorough mixing of the components but only a limited amount of time for the enzyme to be exposed to PAN before gel formation.



PAN (27)

TET = triethylenetetramine

DTT = dithiothreitol

Enz = *Lactobacillus leichmannii* (crude extract)

Scheme 3.14 Immobilisation of N-deoxyribosyltransferase on PAN

The enzyme-containing gel was allowed to stand for 60 min to complete the coupling reaction before being ground into particles and washed with aqueous buffer containing ammonium sulphate to convert residual active

ester groups to amides. Finally, the immobilised enzyme activity was assayed. To maximise the ease of separation of the reaction mixtures from the gel, the gel particles were kept in the centrifuge tubes for the assays and reactions. The initial washings with buffer were collected for analysis of the unbound activity. These initial washings showed a negligible amount of enzyme activity but most was retained in the gel. The immobilised enzyme was assayed every day to measure the retention of activity in the gel.

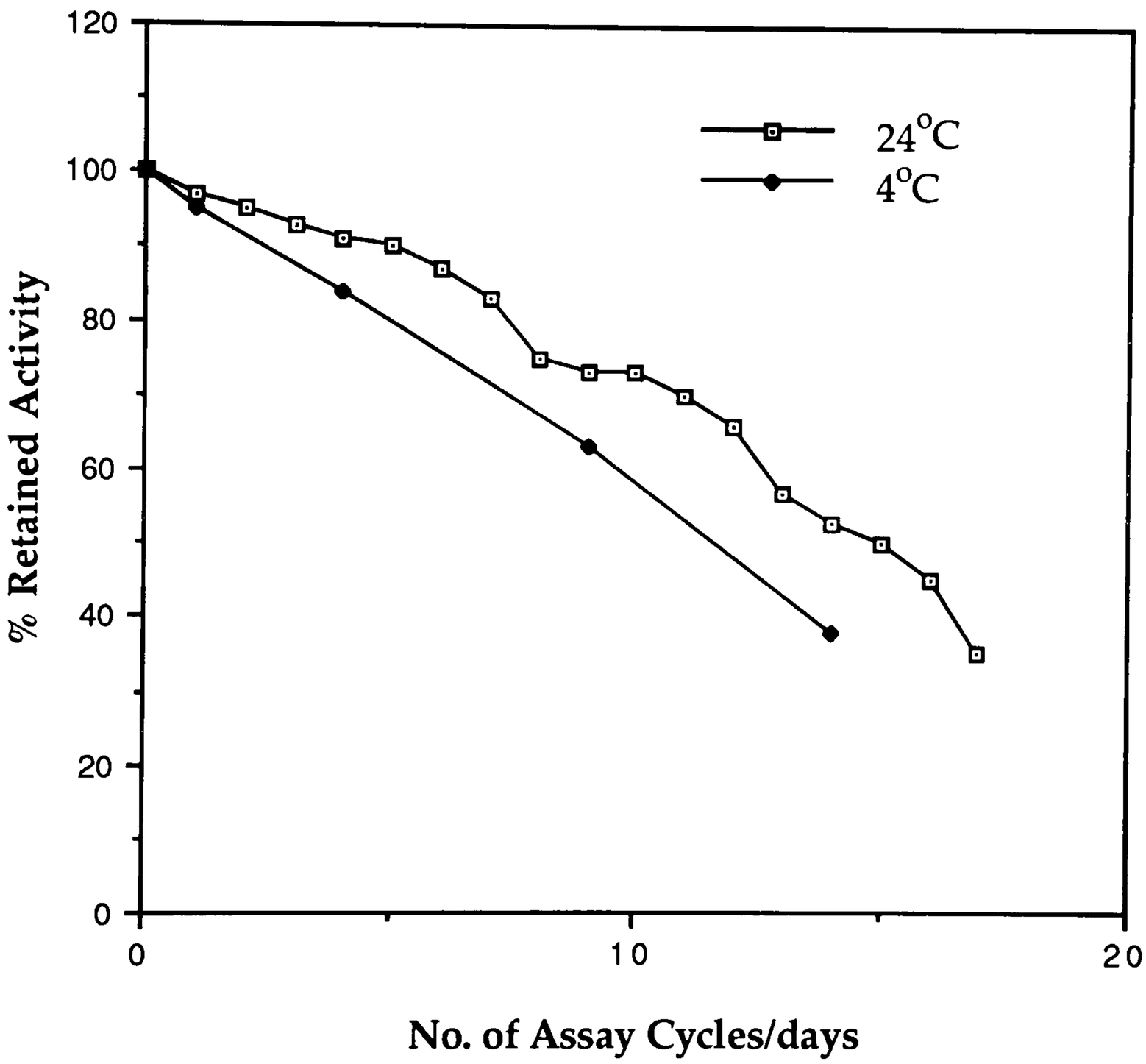


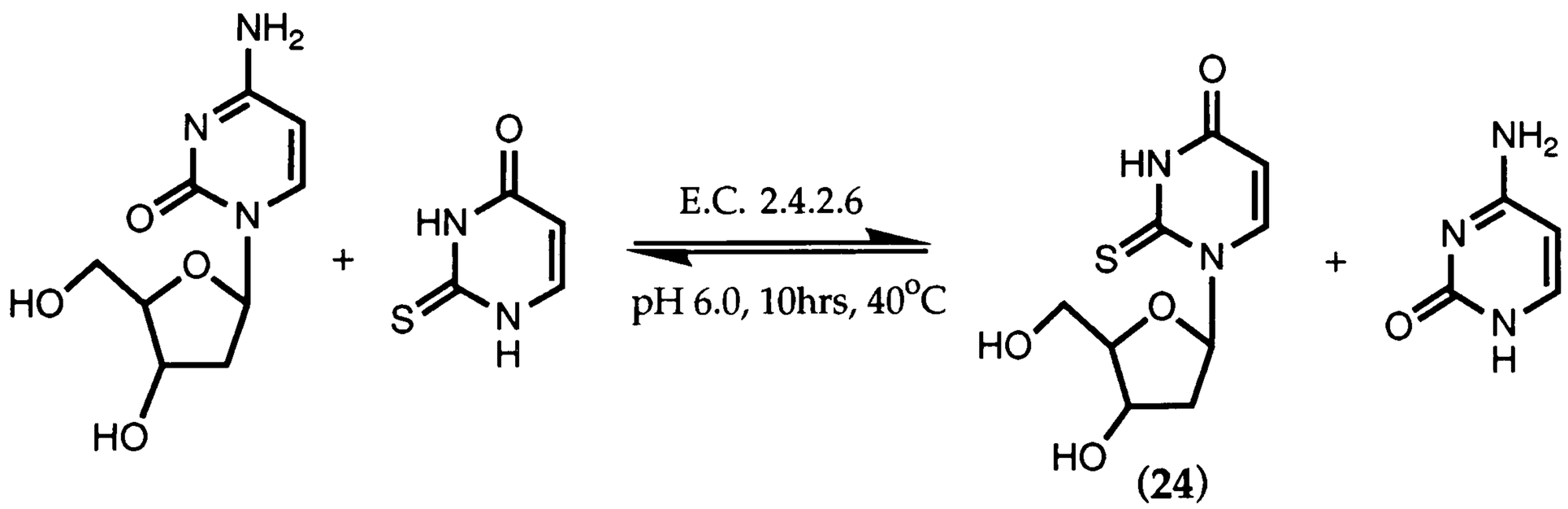
Fig. 3.15 Retention of N-deoxyribosyltransferase on PAN

Considerable activity appeared to be retained in the PAN gel. The immobilised enzyme was subjected to numerous assay cycles; it was found that initially the levels of activity were very high for the first 7 days (≥80%), after this the activity slowly decreased to 50% and then there was a much more rapid loss of activity. A sample of PAN immobilised enzyme

was also stored at 4°C and fractions were removed periodically to assay the activity. As can be seen on the graph the losses in activity showed a similar pattern to that produced by the continuous assay except that the decrease in activity was more rapid. The levels of retained activity could have been affected by several factors: losses in activity might have been due to the elution of the active enzyme from the PAN gel, but this was only a minor contributing factor as negligible amounts of protein were found in the initial washings or the eluted reaction mixtures; any losses in activity were more likely to have been caused by deactivation of the N-deoxyribosyltransferase retained in the gel. As was postulated for the loss in activity seen for the octyl Sepharose, this deactivation may be caused by the dissociation of the subunits of this hexameric enzyme.

3.2.4.5: Synthesis of 2-Thio-2′-deoxyuridine (24) using PAN Immobilised Enzyme

A large scale immobilisation of N-deoxyribosyltransferase on PAN-550 was performed as described in the Experimental section and this was used in the synthesis of the nucleoside analogue, 2-thio-2′-deoxyuridine (**24**).



Scheme 3.16 Synthesis of 2-thio-2′-deoxyuridine (**24**)

Thymidine, the glycosyl donor, and 2-thiouracil, the acceptor base, were thermostated in citrate buffer (pH 6.0) at 40°C. The reaction was followed by reverse-phase HPLC and had reached equilibrium after 10 h. The purified white crystals were identified by ^{1}H NMR and MS (CI) and were compared with the sample prepared using the soluble enzyme. The yield (35%) was comparable with that obtained using soluble enzyme but the use of immobilised enzymes enabled the reaction to be repeated several times, with the same batch of enzyme, to synthesise a large amount of 2-thio-2′-deoxyuridine (**24**).

3.2.5: Conclusion

Immobilisation of N-deoxyribosyltransferase on octyl Sepharose and PAN–550 gel were both successful methods however, the PAN method gave higher and more sustained levels of activity, was easier to handle and incubate, and provided a general batch reactor method for the synthesis of nucleosides. Once the starting PAN is prepared, it is just as convenient to immobilise the enzyme on the gel as it is to absorb it to the Sepharose. From the investigations it was concluded that, because of the ease of preparation of the immobilised enzymes, synthesis of the immobilised enzymes when required, rather than synthesising large batches to store, was the preferred method.

3.3: MATERIALS

Phenyl and octyl Sepharose CL-4B were purchased from Pharmacia. Trityl agarose was purcased from Miles-Yeda Ltd. The filters for the κ-carrageenan filtration unit were purchased from Millipore. κ-Carrageenan, Type III from *Eucheuma cottonii* (containing 0.7% Na, 3.6% K, 2.9% Ca),

was purchased from Sigma. N-Deoxyribosyltransferase (EC 2.4.2.6) was prepared from *Lactobacillus leichmannii* as described in Chapter 2. The reverse phase HPLC was performed on a Techsphere 5C8 column (25cm x 4.6 mm and a precolumn, 5cm x 4.6mm) form HPLC Technology Ltd, Macclesfield, Cheshire, UK.

3.4: EXPERIMENTAL

3.4.1: Standard Assay of Immobilised Nucleoside Deoxyribosyltransferase

The standard reaction assay mixture containing 2′-deoxycytidine and adenine is described for each immobilisation method. After the described time the reaction mixture was separated from the immobilised enzyme and a 20µl aliquot of the assay mixture was applied to an HPLC column. The rate of deoxyribosyl transfer was measured by reverse phase HPLC (as described in Chapter 2). The samples were eluted from the column using a mobile phase of acetonitrile : double distilled water (5:95) at a flow rate of 1.2 ml/min and detected by UV at 254nm.

3.4.2: Immobilisation on Hydrophobic Sepharoses

Phenyl, trityl, and octyl Sepharose were used to immobilise a crude preparation of N-deoxyribosyltransferase. Each gel was poured into a small disposable column (4.5cm x 0.8cm). Each column was washed several times with 10 bed volumes (≥ 35 ml) of citrate buffer (0.05M, pH 6.0) to remove any organic storage solvents. The enzyme (200µl, 2.4mg protein, 2U) was pipetted onto each respective column, allowed to flow onto the Sepharose. The column was then stoppered and left to stand at room

temperature for 10 min. The columns were washed with 2-3 bed volumes of citrate buffer (10ml) before the reaction mixture for the enzyme assay was loaded onto them. This assay mixture contained 2'-deoxycytidine (3.0mM) and adenine (1.0mM), in citrate buffer (10.0mM, pH 6.0). The final volume of the reaction mixture was 1.0ml and it was incubated at 40°C. After 30 min at room temperature the reaction mixture was eluted from the column and the reaction analysed by reverse-phase HPLC immediately and after three hours of being removed from the column. The columns were washed with buffer (10ml) before another sample of the reaction mixture was loaded. These washings were also assayed for enzyme activity. These steps were repeated until no activity was shown to be remaining.

3.4.2.1: Immobilised Enzyme Stability at 4°C

Octyl Sepharose showed good retention of activity and so a sample was stored at 4°C and removed periodically to assay.

3.4.3: Entrapment using κ-Carrageenan

An aqueous solution of κ-carrageenan (4.4%, w/v) was prepared by heating the mixture of powder and distilled water to 60°C to dissolve the polysaccharide. The solution was maintained at 40°C and the enzyme solution (200µl, 2.4mg protein, 2U) was added, and mixed thoroughly. The warm κ-carrageenan and enzyme solution was hardened by the addition of KCl solution (0.3M, 30ml). The mixture was left to harden in the KCl solution for one hour. The resulting solid sheet was cut into small cubes which were placed in a protein filtration unit to produce a batch reactor.

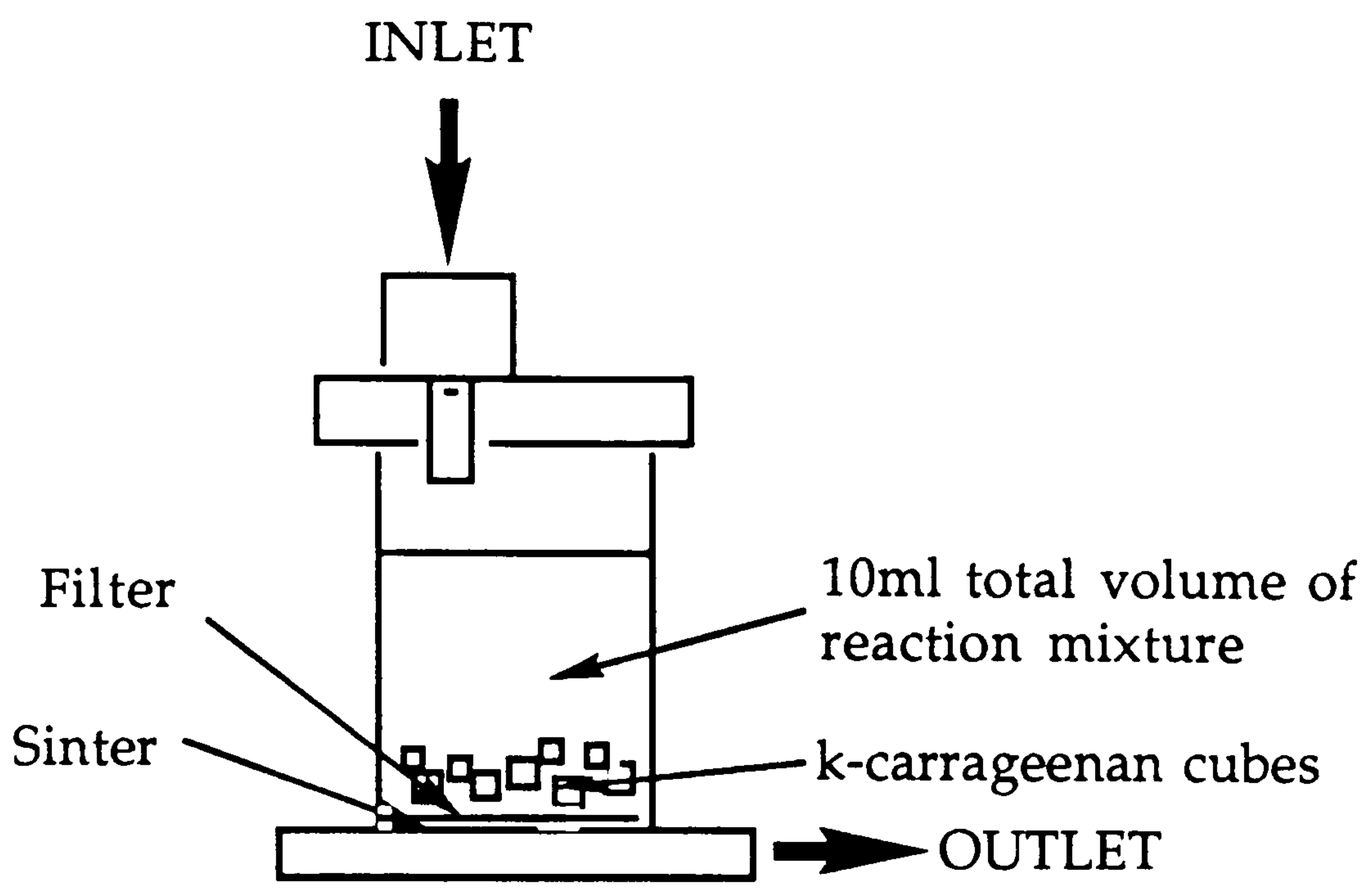


Fig. 3.16 Batch reactor containing κ-carrageenan

The cubes were then assayed for their enzyme activity. The assay reaction mixture, containing 2'-deoxycytidine (30.0mM) and adenine (10.0mM), in citrate buffer (10.0mM, pH 6.0), final volume 10.0ml, was added to the reactor. After 30 min at room temperature the reaction mixture was eluted from the reactor and the assay mixture analysised by reverse-phase HPLC immediately and after three hours of being eluted from the reactor. The κ-carrageenan cubes were washed with buffer (10ml, 1.0mM. pH 6.0) before the next reaction mixture was added to the reactor. The KCl solution and the washings were also assayed for enzyme activity. These steps were repeated until no activity was shown to be remaining.

3.4.4: Poly(acrylamide-co-N-acryloxysuccinimide) (PAN) (27)

The PAN was synthesised as described by Pollak *et al.*[175].

3.4.4.1: Synthesis of N-acryloxysuccinimide (25)

This was carried out by the method of Pollak *et al*[175] to give 61.5g (73% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.80 (4H, s, 2 x CH_2), 6.11 (1H, dd, J= 0.9, 10.7Hz, H_{trans}), 6.26 (1H, dd, J=10.7, 17.3Hz, H_{gem}), 6.63 (1H, dd, J=0.9, 17.3Hz, H_{cis}); ^{13}C NMR (400MHz, $CDCl_3$) δ 25.44, 122.76, 136.10, 160.91, 168.95; IR (Nujol, cm^{-1}) 870, 995, 1260, 1735, 1775, 1800.

3.4.4.2: Synthesis of Poly(acryamide-co-N-acryloxysuccinimide) (PAN) (27)

This was carried out by the method of Pollak *et al*[175] to give 31.5g (100% yield): IR (Nujol, cm^{-1}) 1070, 1210, 1660, 1730, 3200, 3340.

3.4.4.3: Assay for the Active Ester Content of PAN

PAN (~50mg, ~50µmol of active ester groups, dried under vacuum (0.2mm Hg) at 45°C for 24 h) was dissolved and made up to volume in distilled water in a 5ml volumetric flask. A 50µl aliquot of this solution was added into a 5ml quartz cuvette containing Hepes buffer (3000µl 0.1M, pH 7.5), propylamine (50µl 1.0M), and mercaptoethanol (10µl, 1.0M). The rate of appearance of N-hydroxysuccinimide was followed spectrophotometrically at 259nm at room temperature. After the reaction was completed (~60 min) and the increase of the absorbance leveled off, the active ester concentration was calculated. The concentration of active ester groups was calculated to be 573µmol gm^{-1}.

3.4.4.4: Determination of the Gel Time

PAN-550 (100mg) and Hepes buffer (500µl, 0.3M, pH 7.5) were added to a beaker, and the polymer was brought into solution within 1 min. A solution of TET (42µl, 0.5M) was added to the stirred solution and the time taken for the polymer to form a gel was measured. The polymer rapidly became viscous and after 2 min the magnetic stirring bar had stopped turning.

3.4.4.5: Immobilisation of N-Deoxyribosyltransferase

PAN-550 (1.0g, 570µmol of active ester groups), and Hepes buffer (4ml, 0.3M, pH 7.5 containing 50mM adenine) were placed in a 50ml beaker containing a small stirring bar. The polymer was dissolved within 1 min and to the magnetically stirred solution was added DTT (50µl, 0.5M), TET (0.7ml, 0.5M). After 15 sec the crude preparation of N-deoxyribosyltransferase (1.0ml, 10mg protein, 7U) was added to the mixture and the mixture set to a transparent, mechanically resilient gel after 3 min. The gel was allowed to stand for 1 h at room temperature to complete the coupling of the enzyme to the polymer. The pale yellow gel was transferred to a small mortar and ground with a pestle for 2 min. Hepes buffer (25ml, 50mM, pH 7.5 containing 50mM $(NH_4)_2SO_4$) was added and the grinding continued for an additional 2 min. This produced small particles which were diluted by another addition of the Hepes buffer/ammonium sulphate solution (25ml). The mixture was transferred to a centrifuge tube and the gel suspension was stirred magnetically for 15 min to destroy any unreacted esters. The gel suspension was separated from the buffer solution by gentle centrifugation (~3000rpm). The supernatant buffer was assayed for the nonimmobilised N-

deoxyribosyltransferase activity. The assay mixture, containing 2'-deoxycytidine (3.0mM) and adenine (1.0mM) in citrate buffer (10.0mM, pH 6.0), final volume 1.0ml, was added to some of the supernatant buffer (10.0ml, adjusted to pH 6.0). The reaction mixture was incubated at 40°C for 30 min, after which time it was analysed by reverse-phase HPLC. The gel particles were washed with citrate buffer (2x50ml, 10mM, pH 6.0) and then assayed for enzyme activity. The assay reaction mixture, containing 2'-deoxycytidine (30.0mM) and adenine (10.0mM) in citrate buffer (10.0mM, pH 6.0), final volume 50.0ml, was added to the gel particles in the centrifuge tube. After 30 min at 40°C the reaction mixture was separated from the gel particles by gentle centrifugation and the reaction analysed by reverse-phase HPLC immediately and after three hours. The gel particles were then washed with citrate buffer (2 x 50ml, 10mM, pH 6.0) before the next assay mixture was added. The washings were also assayed for enzyme activity. The assays were continued for several days to follow the level of retained activity.

3.4.4.6: Immobilised Enzyme Stability at 4°C

A sample of immobilised enzyme as prepared above was stored at 4°C. Fractions were removed periodically to analyse for enzymatic activity.

3.4.4.7: Large Scale Immobilisation of N-Deoxyribosyltransferase on PAN-550

A suspension of PAN-550 (3.0g, 1710µmol of active ester groups) in Hepes buffer (10ml, 0.3M, pH 7.5 containing 50mM thymidine) was dissolved within 1 min. A magnetic stirrer was added to the polymer solution which was stirred for 30 sec before solution of DDT (150µl, 0.5M) and TET (2.0ml,

0.5M) was added with vigorous stirring, immediately followed by the addition of the crude preparation of N-deoxyribosyltransferase (3.0ml, 36mg protein, 25U). The mixture set to a transparent, resilient gel in 2 min and was allowed to stand for 1 h at room temperature. The gel was transferred to a mortar and ground with a pestle for 5 min. Hepes buffer (100ml, 50mM, pH 7.5 containing 50mM $(NH_4)_2SO_4$)) was added and the grinding continued for an additional 5 min. This produced small particles which were diluted by another addition of the Hepes buffer/ammonium sulphate solution (100ml). The mixture was transferred to centrifuge tubes and the gel suspension was stirred magnetically for 15 min. The gel suspension was separated from the buffer solution by gentle centrifugation (~3000rpm). The gel particles were washed with citrate buffer (3x100ml, 10mM, pH 6.0) and then a small sample was assayed once for enzyme activity (as described for the small scale immobilisation). This preparation of immobilised enzyme was used for the large scale preparation of some nucleosides.

3.4.4.8: Synthesis of 2-Thio-2′-deoxyuridine (24) using PAN Immobilised Enzyme

Thymidine (805mg, 3.54mmol) and 2-thiouracil(150mg, 1.18mmol) were added to citrate buffer (100ml, 1.0mM, pH 6.0) in a centrifuge tube. The mixture was thermostatted at 40°C before the addition of the PAN-550 immobilised N-deoxyribosyltransferase (10ml, 90mg protein ~8U). The centrifuge tube was placed in a pre-warmed stirrer bath at 40°C and the reaction followed by reverse-phase HPLC. The reaction had reached equilibrium after 10 h, therefore the gel suspension was separated from the reaction mixture by gentle centrifugation (~3000rpm). The remaining gel suspension was washed with citrate buffer (3x100ml) to remove all of

the reaction mixture. The above reaction was repeated using the same sample of immobilised enzyme. The volume of the supernatant from the first reaction was reduced *in vacuo* and then lyophilised before being purified by silica flash chromatography (eluted with CH_2Cl_2/MeOH, 18/1) to give 100mg (35% yield) of a white crystalline solid: Rf = 0.17 (CH_2Cl_2/MeOH, 9/1); ^1H NMR (CD_3OD) δ 2.20 (1H, ddd appears as sextet, J=12.8, 9,03, 6.14Hz, $H_{2'a}$), 2.57 (1H, ddd appears as dt, J=6.03, 5.08Hz, $H_{2'b}$), 3.68 (1H, dd, J=4.42, 12.6Hz, $H_{5'b}$), 3.79 (1H, dd, J=3.07, 12.8Hz, $H_{5'a}$), 4.02 (1H, ddd appears as q, J=3 x 3.91Hz, $H_{4'}$), 4.29 (1H, ddd appears as q, J=3 x 4.54Hz, $H_{3'}$), 6.35 (1H, dd, J=6.02, 8.99Hz, $H_{1'}$), 6.66 (1H, d, J=7.6Hz, H_5), 8.16 (1H, d, J=7.6Hz, H_6); MS (CI) m/z 128 (75), 243 [8, $(M+H)^+$].

CHAPTER 4

CONFORMATIONAL STUDIES

4.1: Background

4.1.1: Fluorinated Nucleosides

The introduction of fluorine into naturally occuring compounds involved in biochemical processes can produce analogues having significant biological activity and therapeutic value. For example, the replacement of hydrogen or hydroxyl by fluorine can alter the biological properties of nucleosides dramatically and many fluorinated nucleosides have antiviral or anticancer activities[177, 178].

The main reasons for the effect of fluorine on the properties of organic compounds are:

- fluorine and hydrogen have similar van der Waals radii (H = 1.20Å, F = 1.35Å), but differ dramatically in the polarization of their bonds to carbon. Even greater comparison can be made between fluoro- and hydroxyl- groups. The C–F and C–OH bonds are more similar physiochemically (for instance, in their bond length, C–F = 1.39Å, C–O = 1.43Å, and polarizations) than C–F and C–H bonds (C–H = 1.08–1.11Å). The C–F bond is the strongest single bond (C–F = 108kcal/mol, C–H = 98.7kcal/mol, C–O = 85.5kcal/mol). These factors mean that the substitution of F for H in a molecule should not dramatically alter the steric bulk of the molecule, or impose any gross conformational changes;

• the electronegativity of fluorine (F = 4.0, O = 3.5, H = 2.1) can have pronounced effects on the electron distribution in the molecule; affecting both the acidity and basicity of neighbouring groups, dipole moments within the molecule, and overall reactivity and stability. Fluorine has a pronounced electron withdrawing effect, but can have a repulsive interaction also by electron donation. The fluoro- and hydroxyl- groups have different hydrogen bonding capabilities: the hydroxyl group can be both a donor and an acceptor; whereas the fluoro group can act only as an acceptor. Fluorine is a moderately good leaving group and can sometimes be displaced by nucleophiles at or near the active sites of enzymes, with resultant covalent attachment of an organic moiety to the enzyme;

• the C–F bond improves lipophilicity of the molecule and hence its distribution within an organism. The CF_3 is one of the most lipophilic group known.

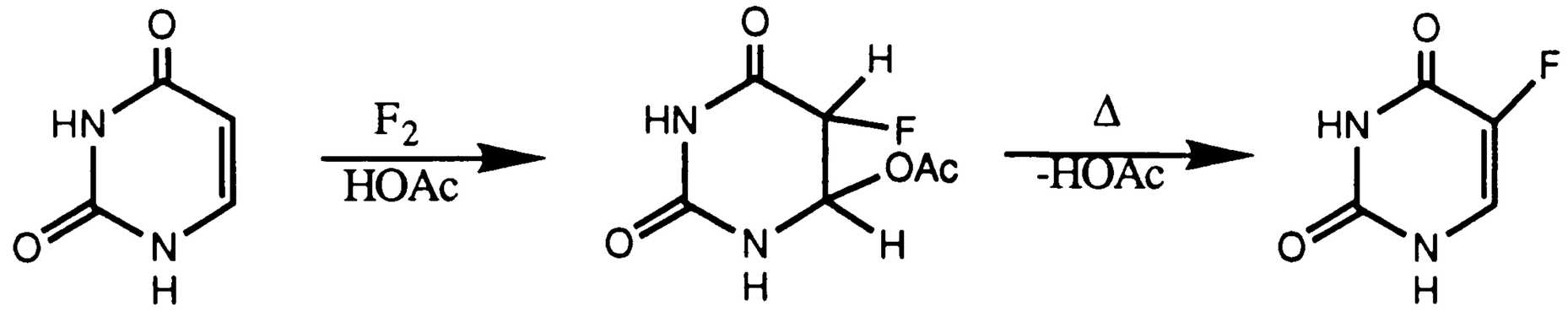
4.1.2: Methods of Fluorination

Due to the interest in fluoro-organic compounds, there are numerous methods for the insertion of fluorine into organic molecules[179, 180], but only the reagents most commonly used for fluorine insertion into nucleosides will be mentioned here:

4.1.2.1: Fluorine

Elemental fluorine is a dangerous oxidizing agent and the gas is usually diluted with nitrogen or argon in the selective fluorination of alkenes in

the presence of other functional groups. The synthesis of 5-fluorouracil is a good example.



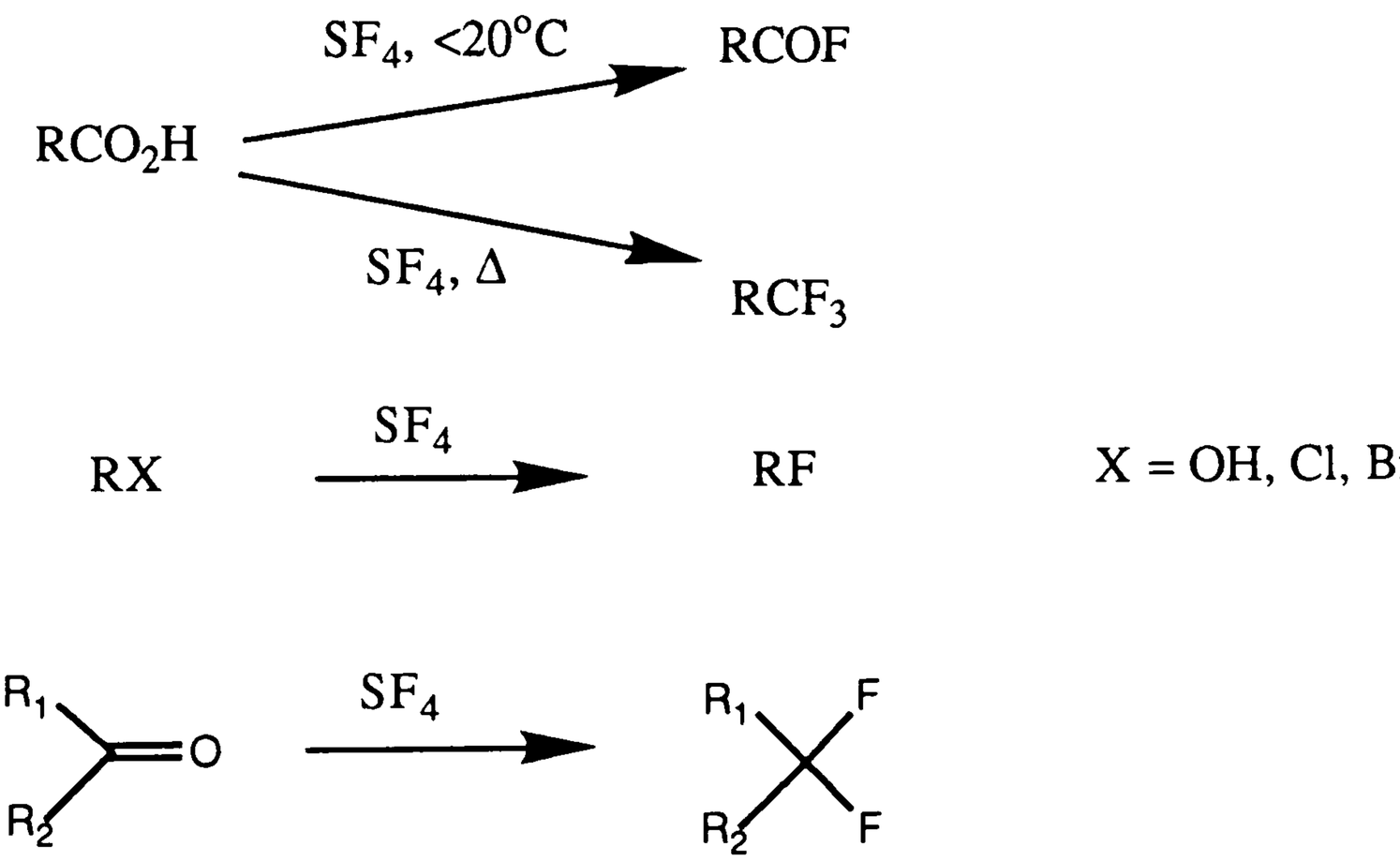
60 – 90% yield

Scheme 4.1 Synthesis of 5-fluorouracil

It was originally proposed that the reaction involved *syn* addition of fluorine, with subsequent solvent-assisted elimination of fluoride, though more recent work suggests the involvement of acetyl hypofluorite (AcOF).

4.1.2.2: Sulphur Tetrafluoride

Sulphur tetrafluoride (SF_4) was first introduced as a fluorinating agent in 1960 and its main uses are:

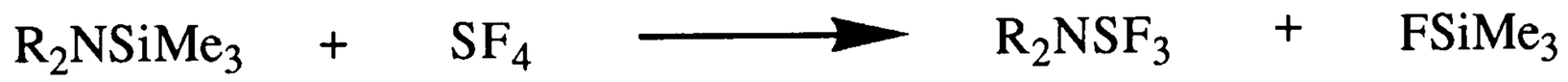


Scheme 4.2 Fluorination with sulphur tetrafluoride

When used in conjunction with Lewis acids or liquid hydrogen fluoride (HF), reaction temperatures and times may often be reduced, and these species usually act catalytically. Reactions of carbonyls with SF_4 almost invariably require more forcing conditions. The major drawbacks of SF_4 are its volatility (b.p. = -38°C) and its toxicity (comparable to phosgene), and reactions often require the use of sealed, stainless steel vessels. The yields of reactions with simple alcohols are usually low and for this reason the modified reagent diethylaminosulphur trifluoride (DAST) is now preferred.

4.1.2.3: Diethylaminosulphur Trifluoride (DAST)

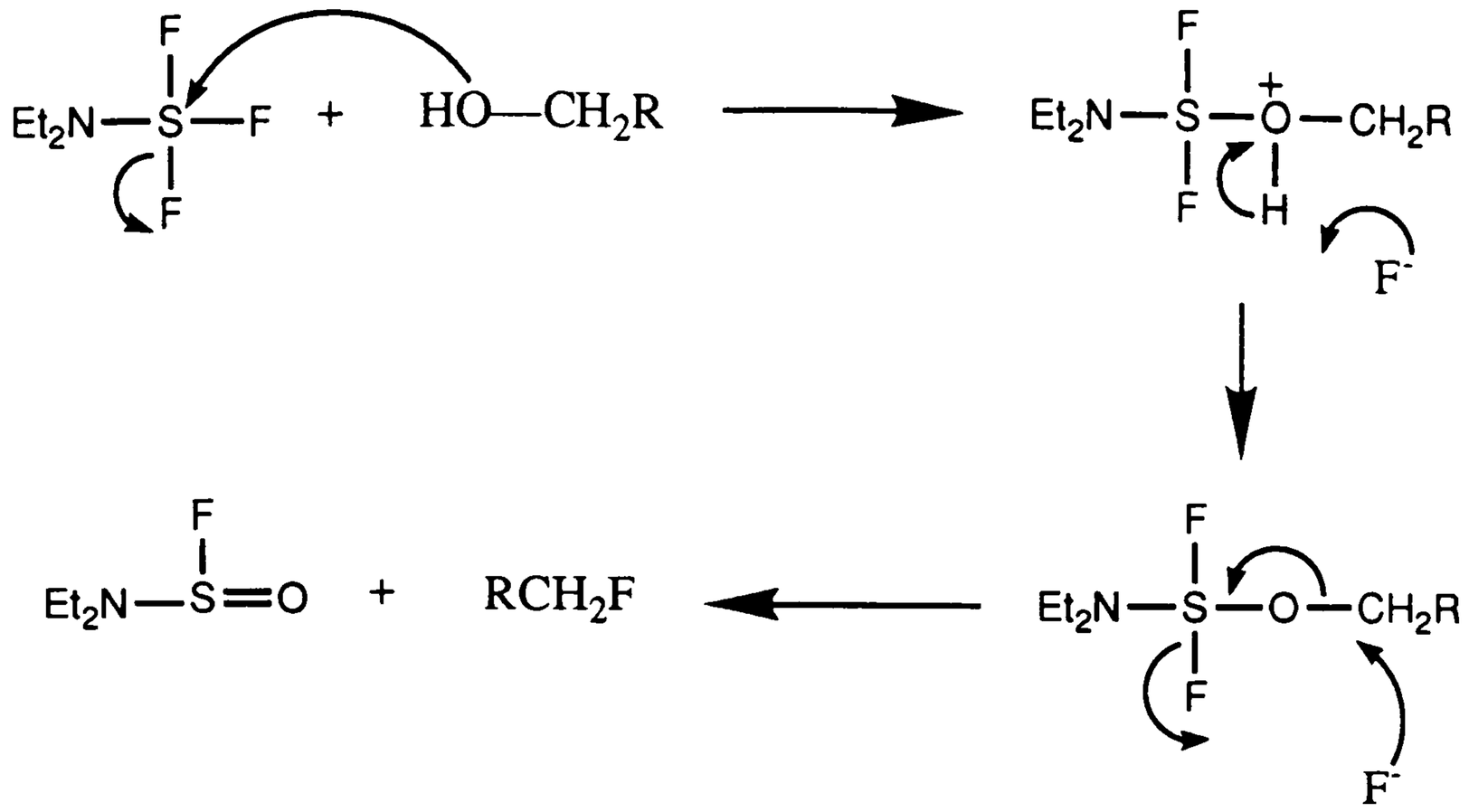
DAST and related reagents were prepared by Middleton[74] by the substitution of one of the fluorine atoms in sulphur tetrafluoride with dialkylamino groups by treating with a dialkylaminotrimethylsilane.



In DAST, R = Et

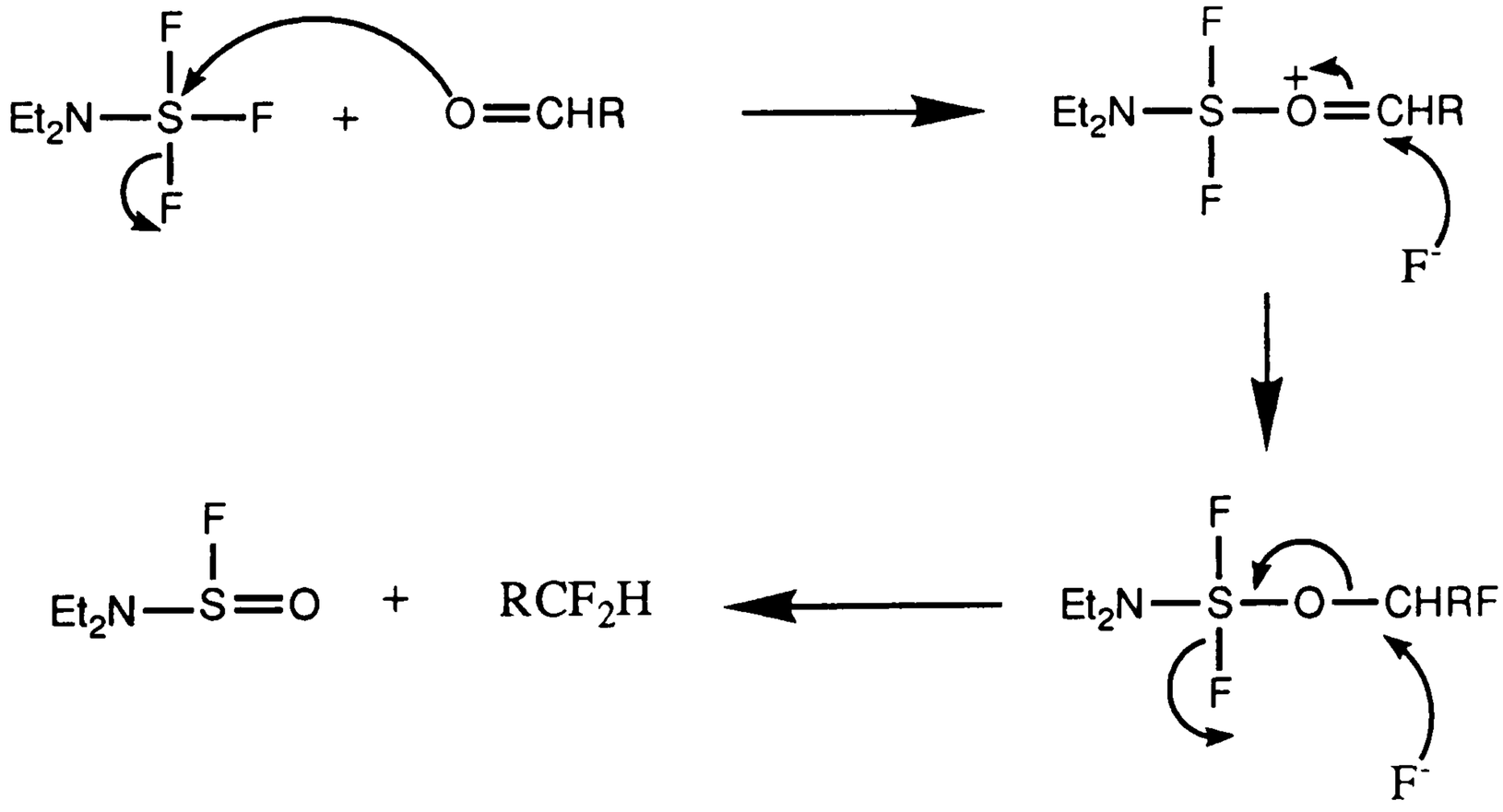
Scheme 4.3 Synthesis of DAST

DAST is relatively easy to handle (b.p. = 46–47°C at 10mmHg) and can be stored in plastic bottles at room temperature or below. The reaction for displacing hydroxyl groups with fluorine, under very mild conditions, is:



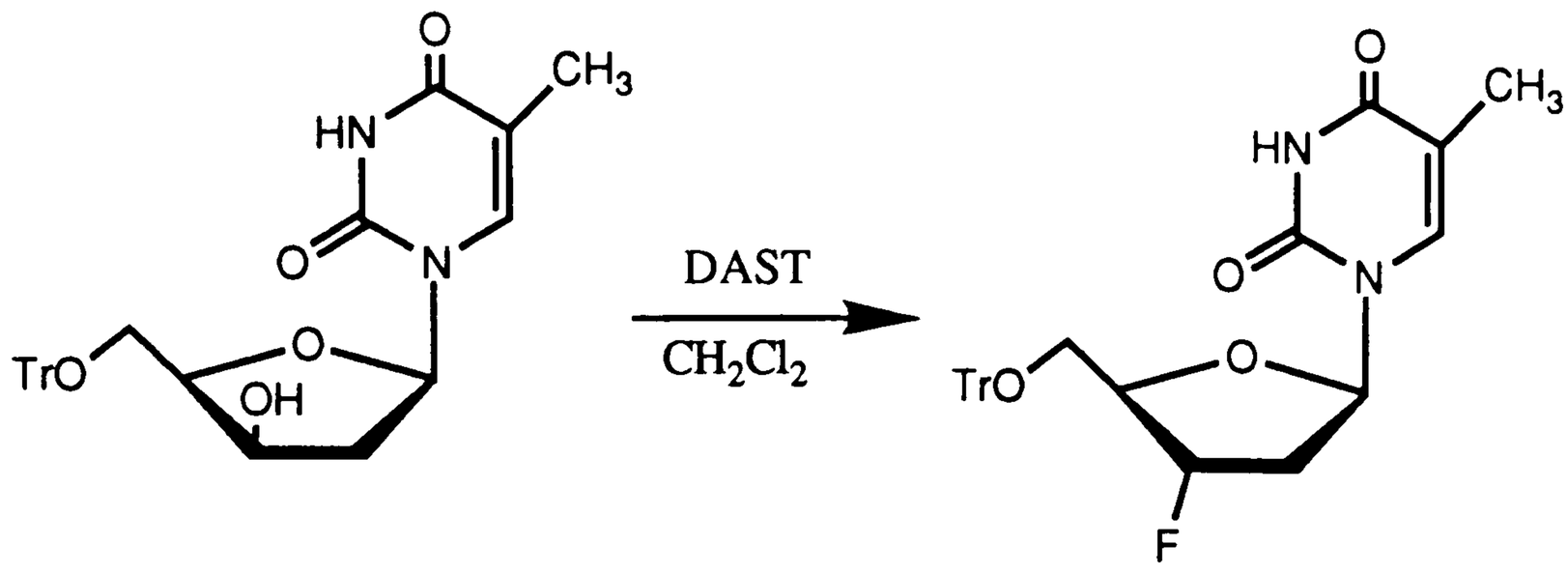
Scheme 4.4 Fluorination of alcohols with DAST

Primary, secondary and tertiary alcohols all react, with high yields of the unrearranged fluoride usually resulting. DAST can also be used for replacing carbonyl oxygens with two fluorine atoms:



Scheme 4.5 Fluorination of carbonyl oxygens with DAST

This reagent is particularly useful for fluorinating acid sensitive compounds. The reactions proceed in good yields using solvents like dichloromethane, and in nucleoside chemistry the reactions usually occur with inversion of stereochemistry.



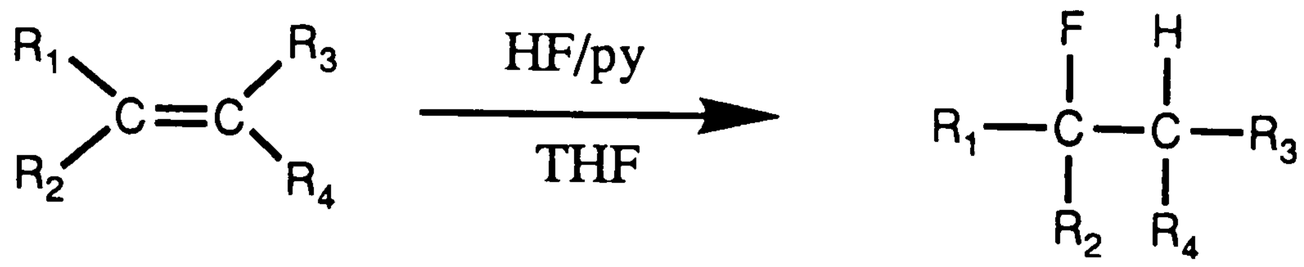
Scheme 4.6 Fluorination of a nucleoside with DAST

Another fluorosulphur reagent is tris(dimethylamino)sulphonium difluorotrimethylsilicate, $(Me_2N)_3S^+Me_3SiF_2^-$, (TASF). This reagent is a hygroscopic solid that is freely soluble in the common organic solvents. Its main use so far has been as a source of fluoride in the displacement of triflates.

4.1.2.4: Hydrogen Fluoride

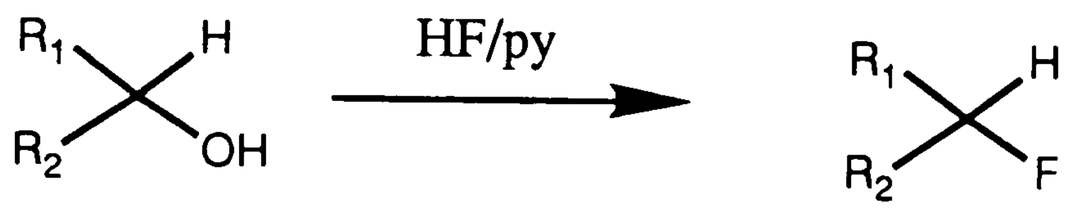
Anhydrous hydrogen fluoride is one of the classical fluorinating agents, but though cheap and readily available its volatility (b.p. = 19.6°C) and corrosiveness provide obstacles to its utility. Its reactions generally require superatmospheric pressure due to its low boiling point. Due to this, the less volative complexes of HF with various n-donor bases have been studied: the introduction of pyridinium poly(hydrogen fluoride), $(HF)_xpy$, a stable liquid of approximate composition 30% pyridine and 70% HF, provided a useful synthetic tool[181].

Alkenes show a typical Markovnikov type addition:



Scheme 4.7 Fluorination of alkenes with HF/py

Reactive tertiary and secondary alcohols are readily fluorinated:



Scheme 4.8 Fluorination of alcohols with HF/py

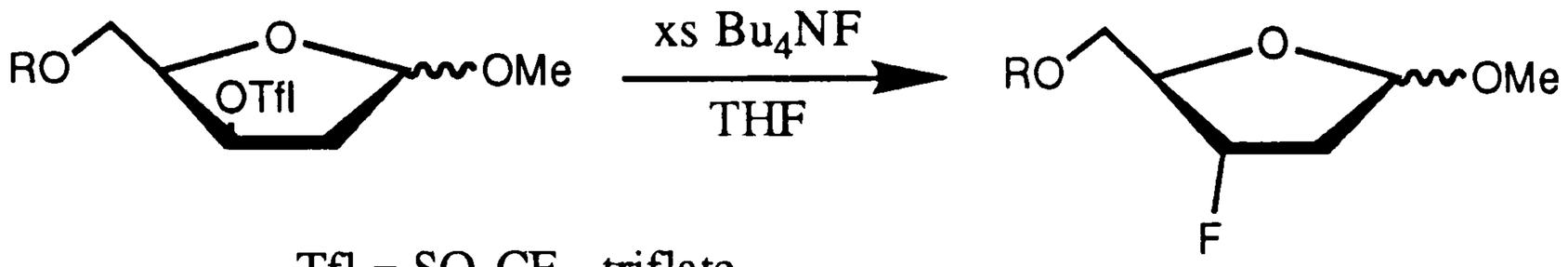
Primary alcohols react only in the presence of added fluoride ions, which indicates that the monomeric fluoride ion is a strong nucleophile, whereas polymeric $F(HF)_x^-$ is a very weak one.

There are also various modified forms of HF/pyridine with improved properties and reactivity in certain reactions.

4.1.2.5: Fluoride

The nucleophilic displacement of an halide ion by a fluoride ion is amongst the cheapest and most widely used methods for the introduction of fluorine. Nowadays, the most common reagents are potassium fluoride, cesium fluoride, potassium fluoride–hydrogen fluoride, and tetralkylammonium fluorides, such as tetrabutylammonium fluoride. The reactions often involve the displacement of good leaving groups such as tosylates, mesylates and triflates. To attain an adequate concentration of dissolved fluoride, anhydrous (water reduces the nucleophilicity of

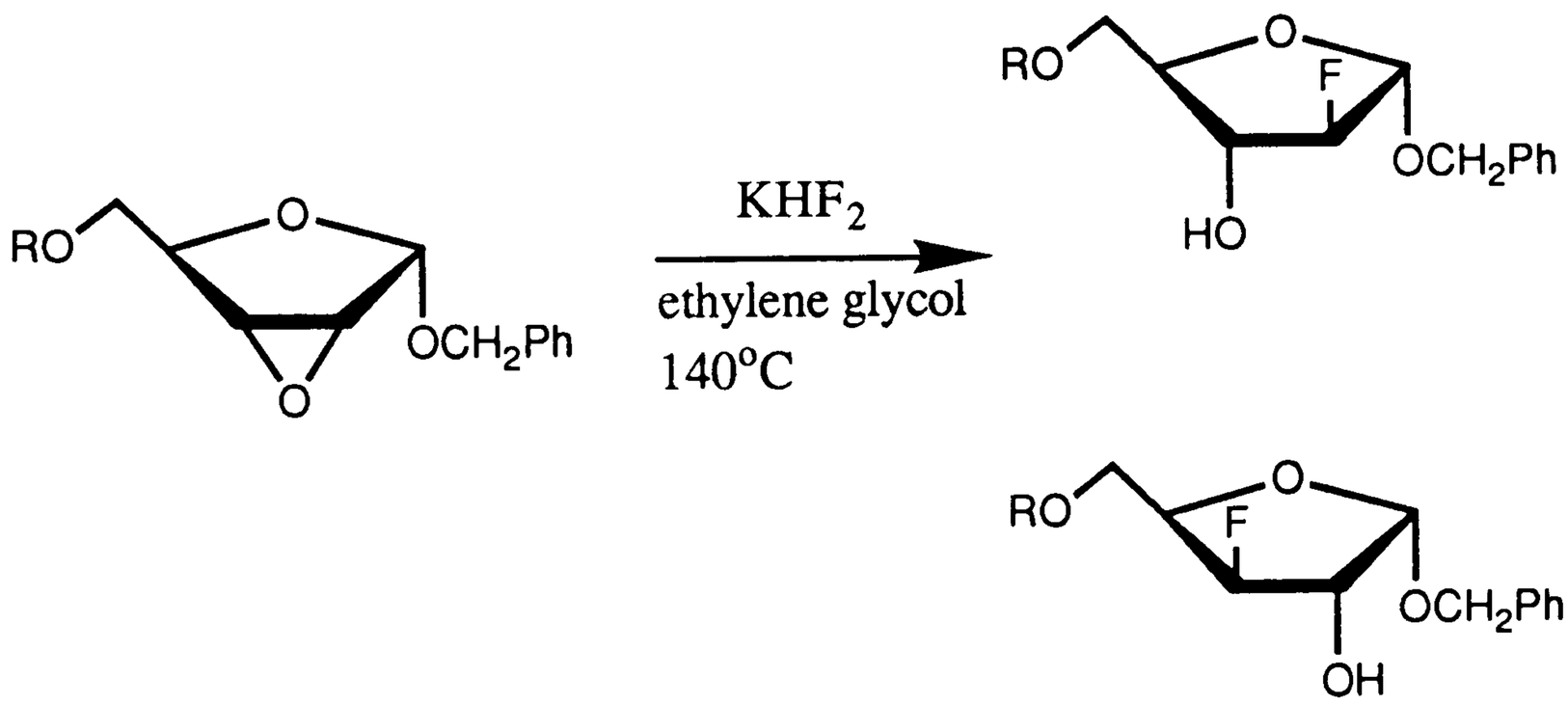
fluoride), polar solvents (CH_3CN, DMF, glycols) are required, though the tetraalkylammonium fluorides can be used in THF and other less polar solvents, as they are more dissociated than other fluoride reagents in organic solvents. Recently, crown ethers with KF in benzene/CH_3CN and polymer-supported fluoride have been used successfully in these S_N2 displacement reactions.



Tfl = SO_2CF_3, triflate

Scheme 4.9 Fluorination of alcohols with tetrabutylammonium fluoride

In addition to the displacement of halides and sulphonates, fluoride reagents can react with epoxides to produce the corresponding fluorohydrins.



Scheme 4.10 Fluorination of epoxides with KF–HF

A number of other reagents are available but all have seen limited use, for example; tetrafluoroboric acid, silver tetrafluoroborate, perchloryl fluoride, hypofluorites, caesium fluoroxysulphate and xenon difluoride.

4.1.3: Synthesis of Fluorinated Nucleosides

Numerous 5-fluorouracil derivatives have been prepared as potential anticancer agents. However, more recently nucleosides containing fluoro sugars have come under scrutiny due to their anticancer and antiviral activities[177, 178]. A large number of fluorinated nucleosides have been evaluated for inhibitory activity against HIV–1 and 3′-fluoro-3′-deoxythymidine has emerged as having the highest antiretroviral potency[48, 182]. Studies were then instigated to see if the cytotoxic effects of 3′-fluoro-3′-deoxythymidine could be reduced by modifying the base moiety of the nucleoside. 5-Chloro-2′,3′-dideoxy-3′-fluorouridine and 4-thio-3′-fluoro-3′-deoxythymidine were found to have improved cytotoxicity[44].

The synthesis of deoxyribonucleosides has been performed enzymatically using N-deoxyribosyltransferases from lactobacilli to catalyse the transfer of a deoxyribosyl residue or an analogue from a donor nucleoside to an acceptor base (as described in Chapter 2). For this reaction 2′-deoxy-, 2′,3′-dideoxy-, and 2′,5′-dideoxynucleosides are efficient glycosyl donors. 3′-Substituted-2′,3′-dideoxynucleosides were studied as possible glycosyl donors for the synthesis of base modified 3′-substituted-2′,3′-dideoxynucleosides. Unfortunately, 3′-substituted-2′,3′-dideoxynucleosides did not act as glycosyl donors in the transfer reaction and so the structures of these nucleosides were investigated to rationalise these results.

Recently, several nucleosides, modified at the C3′-position by a heteroatom such as 2′,3′-dideoxy-3′-thiacytidine ((±)-BCH-189)[183], and (±)-1-(2β,4β)-2-(hydroxymethyl)-4-dioxolanyl)thymine (dioxolane–T)[184] have been reported which show excellent anti–HIV activity *in vitro*.

Dioxolane–T was synthesised in order to investigate the substrate ability of C3′–heteroatom modified sugars in our enzyme system.

4.1.4: Structure–Activity Investigations

The crystal structures of many of the nucleosides and their analogues have been determined and the relationship between their crystal structures and their efficacies as anti–HIV agents has been discussed[163]. However, crystal structures give information on molecular conformations in the solid state and little information can be gained concerning the flexibilities of the molecules and their shapes in solution or in an active site of an enzyme.

4.1.4.1: Structure of Nucleosides

The structure of a nucleoside can be defined by[185]:

- the conformation of the sugar group;
- the conformation at the glycosidic linkage, i.e. the position of the base relative to the sugar group;
- atomic bonding distances and angles within a molecule;
- inter- and intramolecular interactions, especially hydrogen bonding.

The conformations are described using dihedral (torsion) angles. In the atomic group A–B–C–D these angles denote the angle between the bonds A–B and C–D looking along the B–C bond. The dihedral angle is zero when A–B and C–D are *cis*-planar to each other, and is regarded as positive when C–D is twisted clockwise relative to A–B. A dihedral angle is designated *cis* or *trans* when the angle is approximately 0 or 180°

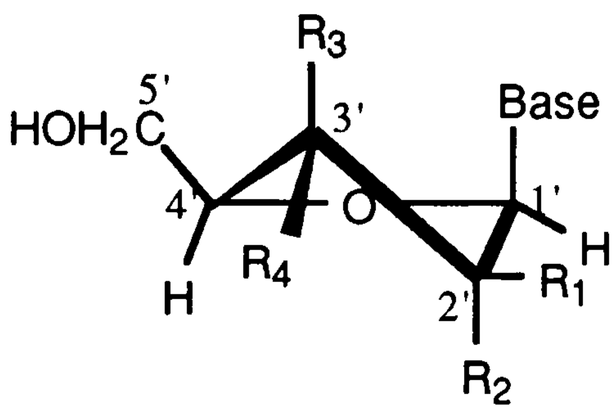
respectively and as (+)*gauche* and (-)*gauche* when the angle is about +60 or -60° respectively.

4.1.4.2: Geometry of the Sugar Group

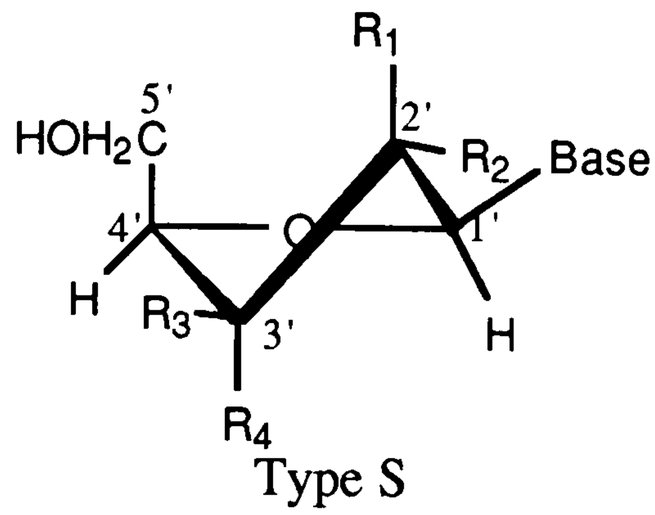
The five-membered sugar ring adopts a puckered conformation because of eclipsing effects. All the bonds are positioned so there will always be unwanted transannular interactions (i.e. interactions between the substituents on C–1 and C–3 or C–1 and C–4). These occur because the internal space is not large enough for all the quasi-axial hydrogen atoms to fit without coming into conflict. The transannular strain can be reduced if the molecule adopts a conformation where some of the C–C bonds are eclipsed or partically eclipsed but this results in Pitzer strain. Therefore, each ring adopts conformations that minimise both sorts of strain as much as possible. For the sugar ring the conformation is either in an envelope, E, or in a half-chair, T, form. Substitution effects lead to preference for those conformations in which the atoms C(2′) and C(3′) are displaced by ~0.5Å from the plane through the other atoms C(4′)–O(4′)–C(1′). Atoms lying on the same side of this plane as C(5′) are designated *endo* and those on the other side *exo*.

It has been shown by X-ray data and NMR studies that the sugar ring can adopt a plethera of conformations. The whole feasible range of sugar conformations has been described by a pseudorotation cycle where the barrier to planarity has been reported to be 5.2kcal/mol. However, the introduction of the N and S type notation by Altona and Sundaralingam[186] has clarified the classification and has shown that two relatively narrow pseudorotational ranges are preferred by the sugar rings. Type N conformers comprise all conformations that occupy the northern

half of the pseudorotational cycle and type S conformers occupy the southern half of the cycle.



Type N
C(2')-exo, C(3')-endo
$^3T_2$

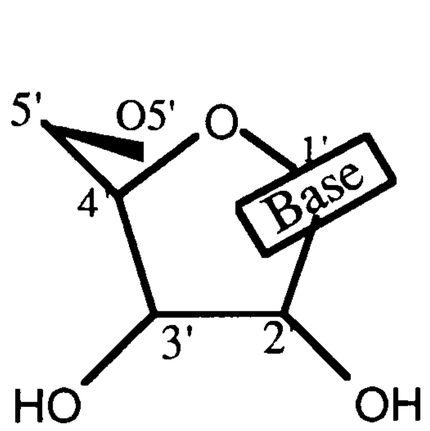


Type S
C(2')-endo, C(3')-exo
$^2T_3$

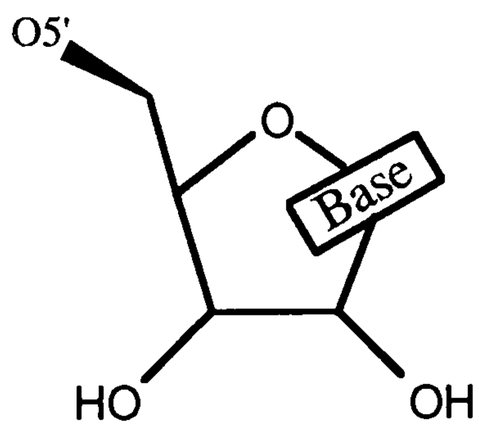
Fig. 4.11 Conformation of the sugar ring

4.1.4.3: Orientation about C(4′)–C(5′)

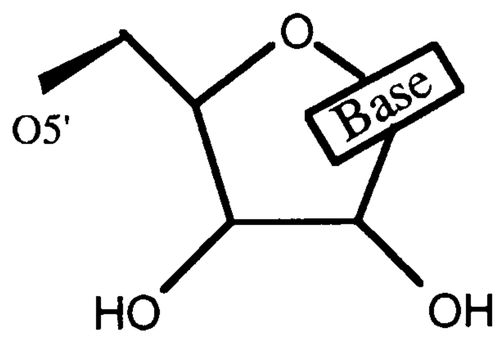
The C(4′)–C(5′) can rotate relative to the sugar ring, assuming three possible conformations, designated *gauche,gauche*, *gauche,trans*, or *trans,gauche* depending upon the angles.



gauche,gauche
-60°, +60°



gauche,trans
+60°, 180°



trans,gauche
180°, -60°

Fig. 4.12 Orientation about C(4′)–C(5′)

In most ribonucleosides and deoxyribonucleosides the preferred orientation is *gauche,gauche* due to the formation of intramolecular O(5′)-- --H–C hydrogen bonding between the sugar and the heterocyclic base. However, in some nucleoside analogues the change in sugar puckering

and the presence of substituents on the sugar or base can cause various effects on the orientation of the C(4′)—C(5′) bond.

4.1.4.4: Orientation about the Glycosidic Bond

Rotation of the base around the glycosidic bond relative to the sugar group is not free but subject to steric hindrance, primarily by the endo proton at C(2′). Thus the nucleosides can exist in two conformations, designated *syn* and *anti* depending upon whether O(2) in pyrimidine nucleosides or N(3) in purine nucleosides lies above the plane of the sugar group or points away from it.

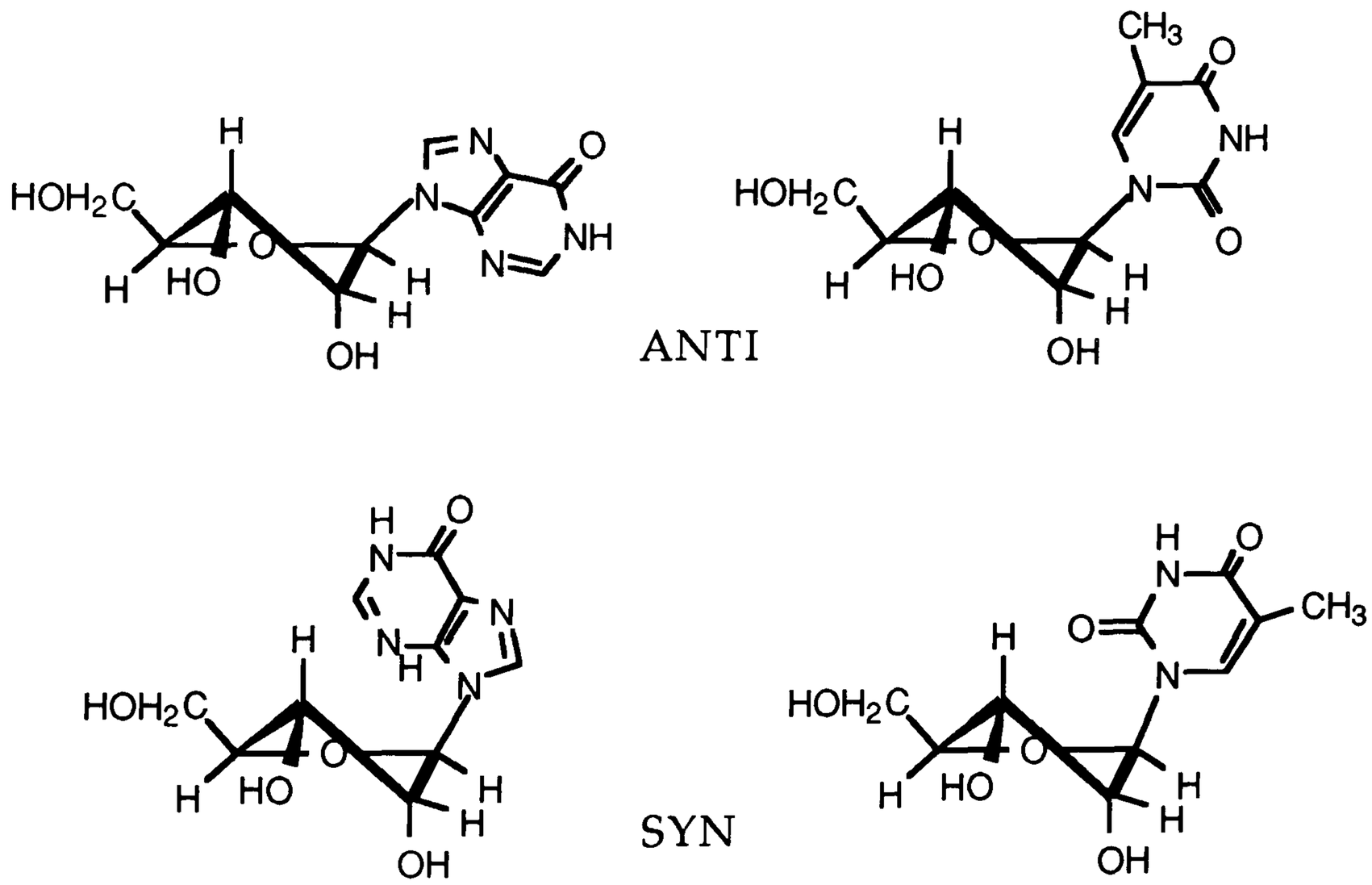


Fig. 4.13 Orientation about the glycosidic bond

The preferred orientation of the heterocyclic base is *anti* but some nucleosides with substituted bases, such as 8-bromoadenosine, do adopt the *syn* conformation.

4.1.4.5: Conformational Analysis by ^{1}H NMR Spectroscopy

The conformation and configuration of a nucleoside are vital determinants of its properties in a biological system. Most of the available structural data has been derived from X-ray crystallographic studies which have been very useful in determining the conformations of nucleosides as described previously. However, the inherent flexibility of nucleosides has stimulated investigations of the structural and conformational behaviour of nucleosides in solution. The most widely used method for structural studies in solution is high-resolution NMR spectroscopy, which can give information on intermolecular interactions, conformational properties and the dynamic behaviour of nucleosides[186-188].

The conformationally significant bonds in a nucleoside are those which exhibit some torsional freedom about the covalent bond linking two atoms. This freedom may be constrained within the limits of a cyclic ring or they may take the form of unhindered rotation about a bond. A typical nucleoside can be viewed as consisting of three components:

- rotation about the glycosidic C–N bond joining the base and sugar rings;
- rotation about the exocyclic groups, especially about the C–4′—C–5′ bond;
- pseudorotation of the sugar ring, where the atoms adopt a variety of ring pucker modes.

This conformational information can be obtain from three NMR parameters:

- chemical shifts (δ), which give information about hydrogen bonding and base-stacking interactions;
- spin-spin coupling constants (J/Hz), which are the most important source of conformational data, due to the dependence of vicinal coupling constant magnitudes on the dihedral angle;
- spin-lattice relaxation times (T_1) and nuclear Overhauser enhancement (nOe) measurements, which are used to resolve conformational features of nucleosides by studying the proximity of certain groups and atoms.

These methods can be used in the determination of the conformational features of nucleosides as outlined previously. Several empirical and semi-empirical methods have been proposed for the evaluation of pentose ring conformational properties from coupling data and of these, the pseudorotation approach of Altona and Sundaralingam[186] is conceptually the most sophisticated. In this approach, the pentose ring is described in terms of two pseudorotation parameters, P, the angle of pseudorotation, and τ, the degree of pucker. From this method, coupled with crystallographic data, it is possible to calculate pseudorotational parameters for all the major conformations of nucleosides. This method also gives a quick way to calculate the conformer populations and related equilibrium constants.

While crystallographic data gives information about the nucleosides in the solid state, it is important to remember that in solution the pentose ring does not possess a unique rigid structure but it is in a dynamical

equilibrium between at least two or more rapidly interconverting puckered conformations which have been termed type N conformers (P = 0 ± 90°), and type S conformers (P = 180 ± 90°).

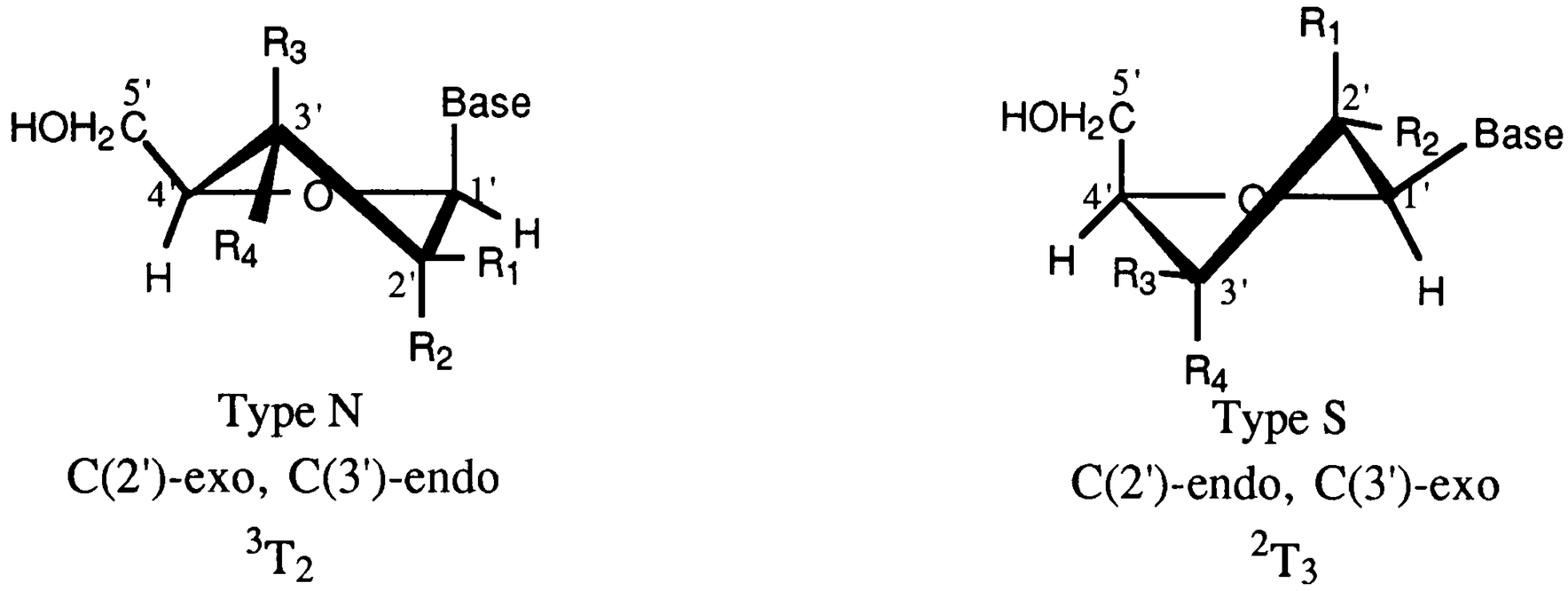


Fig. 4.14 Conformation of N and S sugars

Analysis of the NMR data can lead to information about the conformer populations and the equilibrium constants for the equilibrium $N \rightleftharpoons S$. Studying the variable temperature NMR spectra of nucleosides leads to information about the free energy, enthalpy and entropy of the system. The temperature dependance of the coupling constants can yield quantitative information with regard to the position of the dynamic equilibrium between the principal conformers of the sugar ring.

As the synthesis of 3′-substituted-2′,3′-dideoxynucleosides was not possible by the enzymatic transfer method (as described in Chapter 2), an investigation into the conformations adopted by the nucleosides, which were and were not glycosyl donor nucleosides in the transfer reaction, was undertaken. Little is known about the active sites of the N-deoxyribosyltransferases from lactobacilli, but major factors accounting for this lack of reactivity may be steric hindrance or dipolar effects that inhibit the binding of a substrate to the transferase. Another factor which may be important is the conformation and "flexibility" of the deoxyribose ring, as

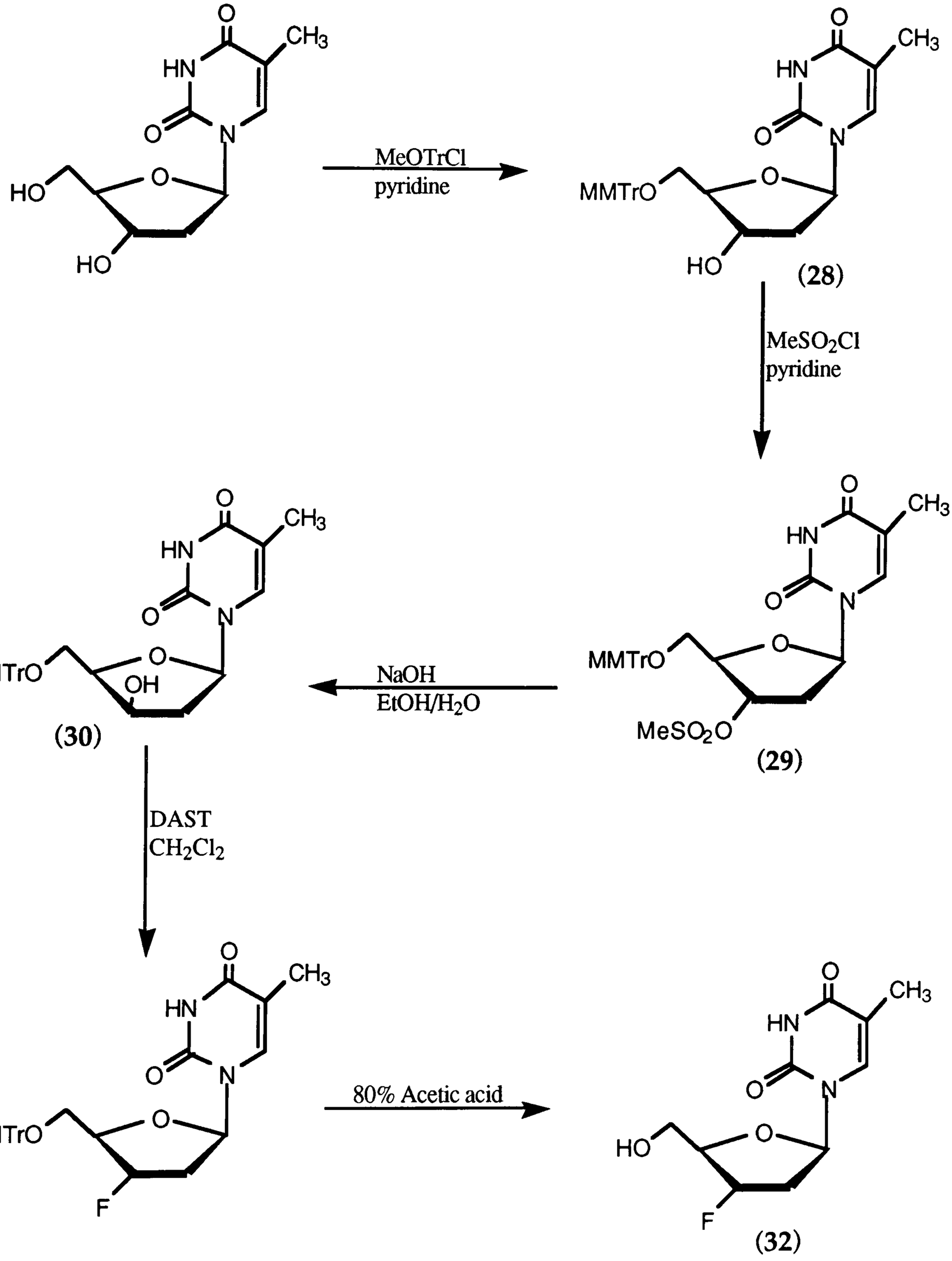
outlined above, because a substrate which adopts a "rigid", unfavourable shape may bind incorrectly, or not bind at all, to the active site of the transferase and may be inactive as a substrate. A number of NMR studies of nucleoside conformation have been reported at constant temperature[186, 188]. Altona and Sundaralingam, and Davies and Danyluk have described methods for calculating, from coupling constants in ^{1}H NMR spectra, the amount of N– and S–character in the favoured conformations of nucleosides in solution. However, little work has been published on the change in conformation of the sugar residues with change in temperature. Slessor and Tracey[189] investigated the conformation of the 2-deoxyribosyl moiety in the naturally occuring nucleosides and found a preference for them to adopt an S–conformation. Information from variable temperature ^{1}H NMR studies can be used to determine the "flexibility" of sugar rings in nucleosides. Therefore, variable temperature NMR spectroscopy was used to investigate the conformations and "flexibility" of the deoxyribose ring in a number of deoxyribonucleosides, with and without substituents on the ring.

4.2: RESULTS AND DISCUSSION

4.2.1: Synthesis of 1-(2′-deoxy-β-D-*erythro*-pentofuranosyl)thymidine (31)[73] and 3′-fluoro-3′-deoxythymidine (32)

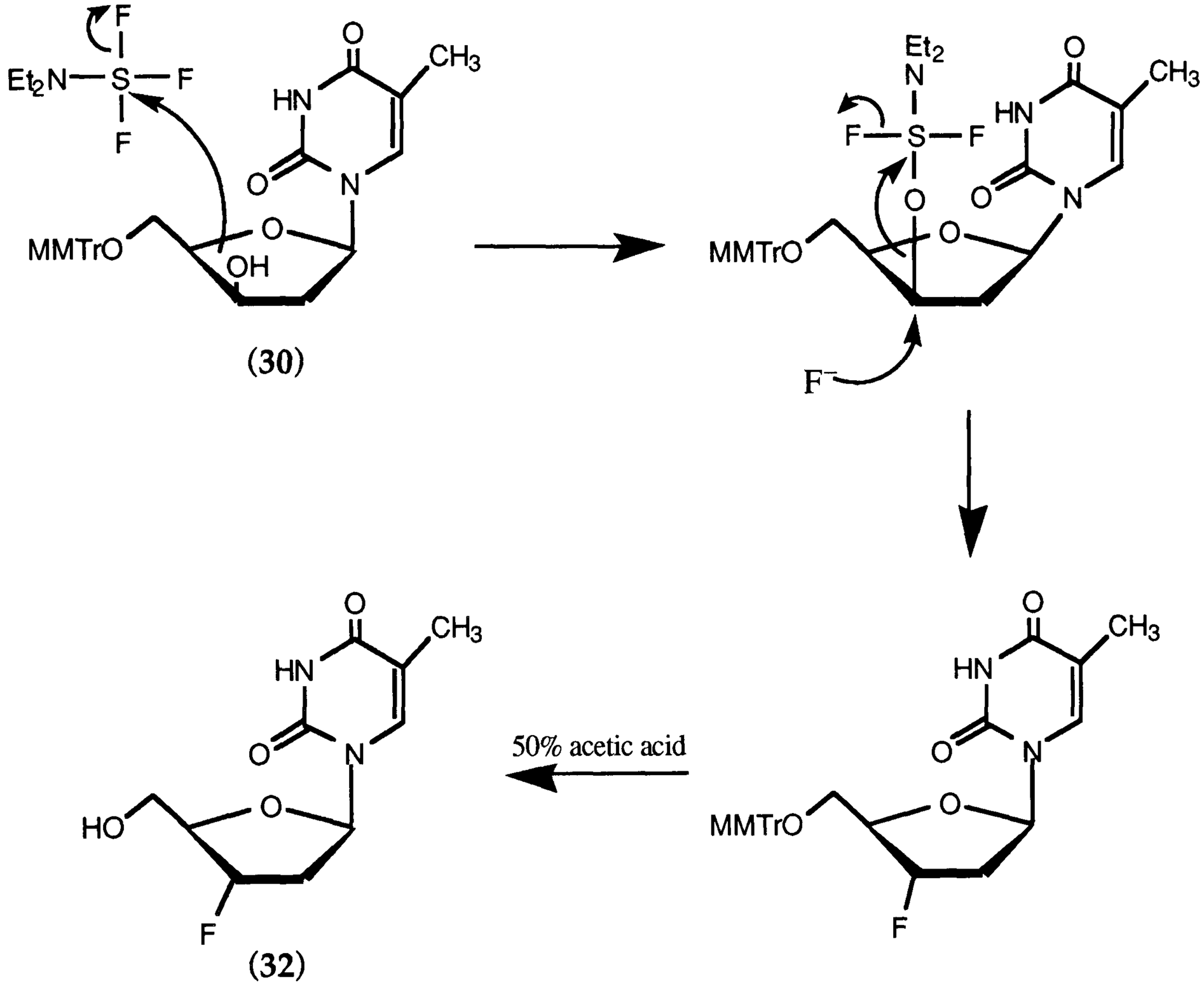
The 5′–hydroxyl monomethoxytritylation of thymidine followed by the 3′–hydroxyl mesylation in the presence of DMAP (as discussed in Chapter 2) gave the required starting material for both syntheses in 69% yield. The monomethoxytrityl protecting group was used rather than trityl as the conditions for its removal are less severe than those for trityl and therefore higher yields of product are obtained. Refluxing 3′-

methanesulphonyl-5′-methoxytritylthymidine (**29**) with excess of alkali afforded the 5′-methoxytrityl derivative of 2′-deoxylyxosylthymine (**30**) via the 2,3′-anhydronucleoside.



Scheme 4.15 Synthesis of 1-(2′-deoxy-β-D-*erythro*-pentofuranosyl)thymidine (**31**) and 3′-fluoro-3′-deoxythymidine (**32**)

This compound could be either demethoxytritylated with acetic acid to afford the 1-(2′-deoxy-β-D-*erythro*-pentofuranosyl)thymidine (**31**) in 57% overall yield, or treated with DAST and then demethoxytritylated with acetic acid to afford 3′-fluoro-3′-deoxythymidine (**32**) in 10% overall yield.



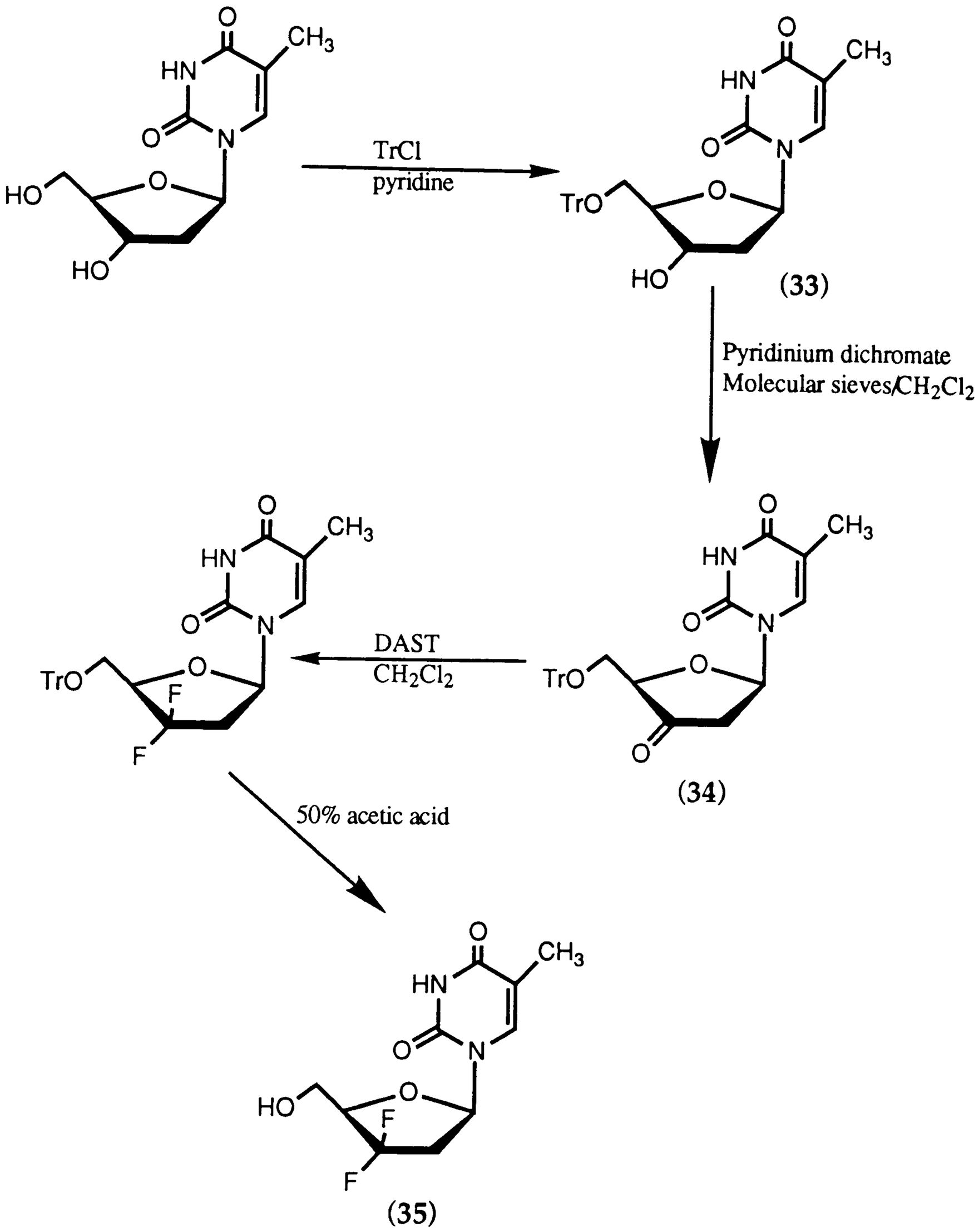
Scheme 4.16 Fluorination of 1-(5′-methoxytrityl-2′-deoxy-β-D-*erythro*-pentofuranosyl)thymine (**30**) with DAST

Although some destruction and demethoxytritylation was observed in the fluorination step, DAST is still the most favoured reagent for fluorination in the presence of acid-labile group (i.e. the glycosidic bond and the monomethyloxytrityl ether). The advantage of this reagent is that products of dehydration and carbonium ion rearrangement are formed in much smaller amounts than with other fluorination agents.

The lone-pair of electrons on the secondary hydroxyl group attack the sulphur centre of DAST and displace a fluoride ion. This converts the hydroxyl group into a better leaving group which is subsequently attacked, with inversion of configuration in an S_N2 displacement reaction, by a fluoride ion to afford the required 3′-exo fluorinated nucleoside.

4.2.2: Synthesis of 3′,3′-difluoro-3′-deoxythymidine (35)[190]

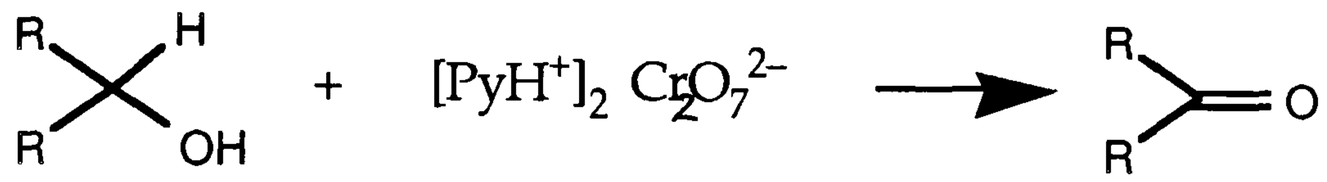
The 5′–tritylthymidine (**33**) was used as the starting material for this synthesis. Oxidation, fluorination and detritylation with acetic acid afforded 3′,3′-difluoro-3′-deoxythymidine (**35**) in 17% overall yield.



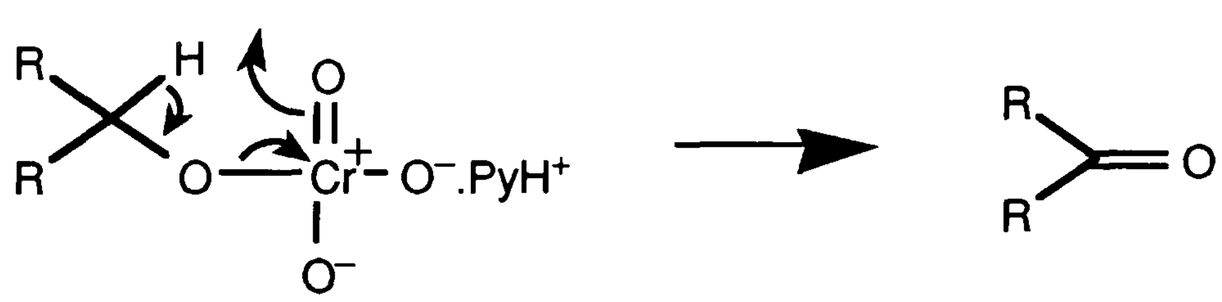
Scheme 4.17 Synthesis of 3′,3′-difluoro-3′-deoxythymidine (**35**)

Oxidation of the 5′-trityl protected thymidine was accomplished in 73% yield using pyridinium dichromate (PDC) as the oxidising agent. This reagent is superior to pyridinium chlorochromate (PCC), which has a mildly acid character and therefore precludes its use with acid sensitive substrates or products. On the contrary, PDC can be used with alcohols and ketones containing acid-sensitive functionality (i.e. the glycosidic bond

and the trityl ether). The 3′-keto-derivative of thymidine has been reported to be quite unstable so careful reaction conditions must be employed to prevent any decomposition occurring. The addition of PDC (1–2mol equiv) and molecular sieve powder (0.5–1.0g per mmol of alcohol) to 5′-tritylthymidine affected a quick and efficient oxidation. The molecular sieves catalyse the reaction by favouring the cleavage of the C–H bond from the alcoholic carbon. There is some controversy as to the mechanism of this reaction but an intramolecular proton transfer is postulated.



Scheme 4.18 Oxidation of alcohols with PDC/molecular sieves



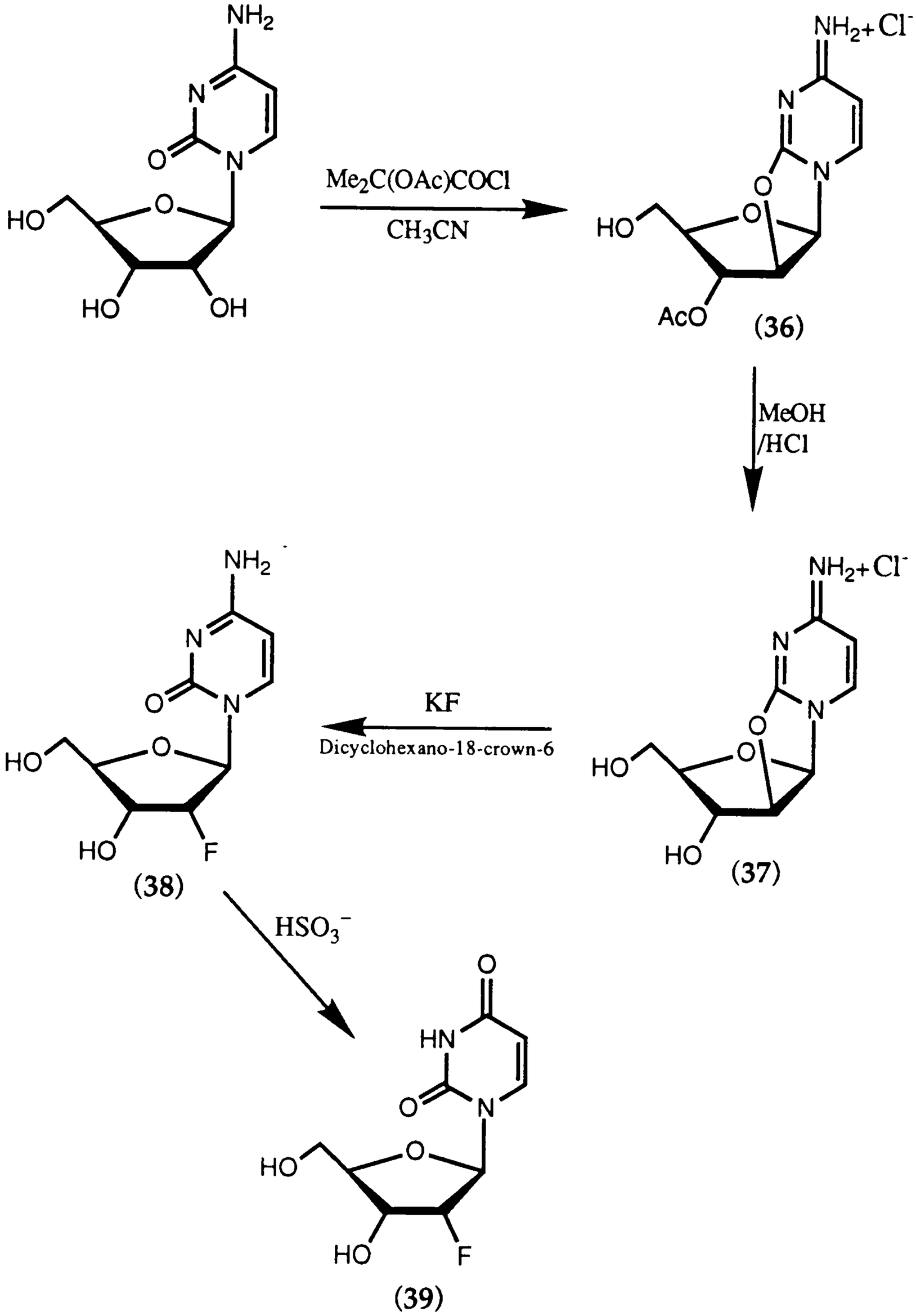
Scheme 4.19 Oxidation of alcohols with PDC/molecular sieves

Oxidation of the alcohols takes place at specific sites on the surface of the molecular sieve. The alcohol is bound to the sieves by the hydroxy function and the molecular sieves orientate the alcohol and the chromium species so that the hydrogen can be transferred. The sieves also stabilise the charges on the intermediate species which facilitates the reaction.

The 3′-keto-5′-tritylthymidine (**34**) was then subjected to DAST in order to prepare the gem 3′,3′-difluoride derivative of thymidine. The lone-pair of electrons on the keto group attack the sulphur centre of DAST and displace a fluoride ion. This produces a trivalent, positively charged oxygen centre which is neutralised by the attack of a fluoride ion on either the α– or the β– face of the sugar ring to afford the monofluorinated nucleoside. The ketone is thus converted into the same species as in the displacement of hydroxyl groups with DAST. This good leaving group is subsequently attacked, with inversion of configuration in an S_N2 displacement reaction, by a fluoride ion to afford the required gem-3′,3′-difluorinated nucleoside (**35**) in 16% overall yield.

4.2.3: Synthesis of 2′-deoxy-2′-fluorouridine (39)[191-193]

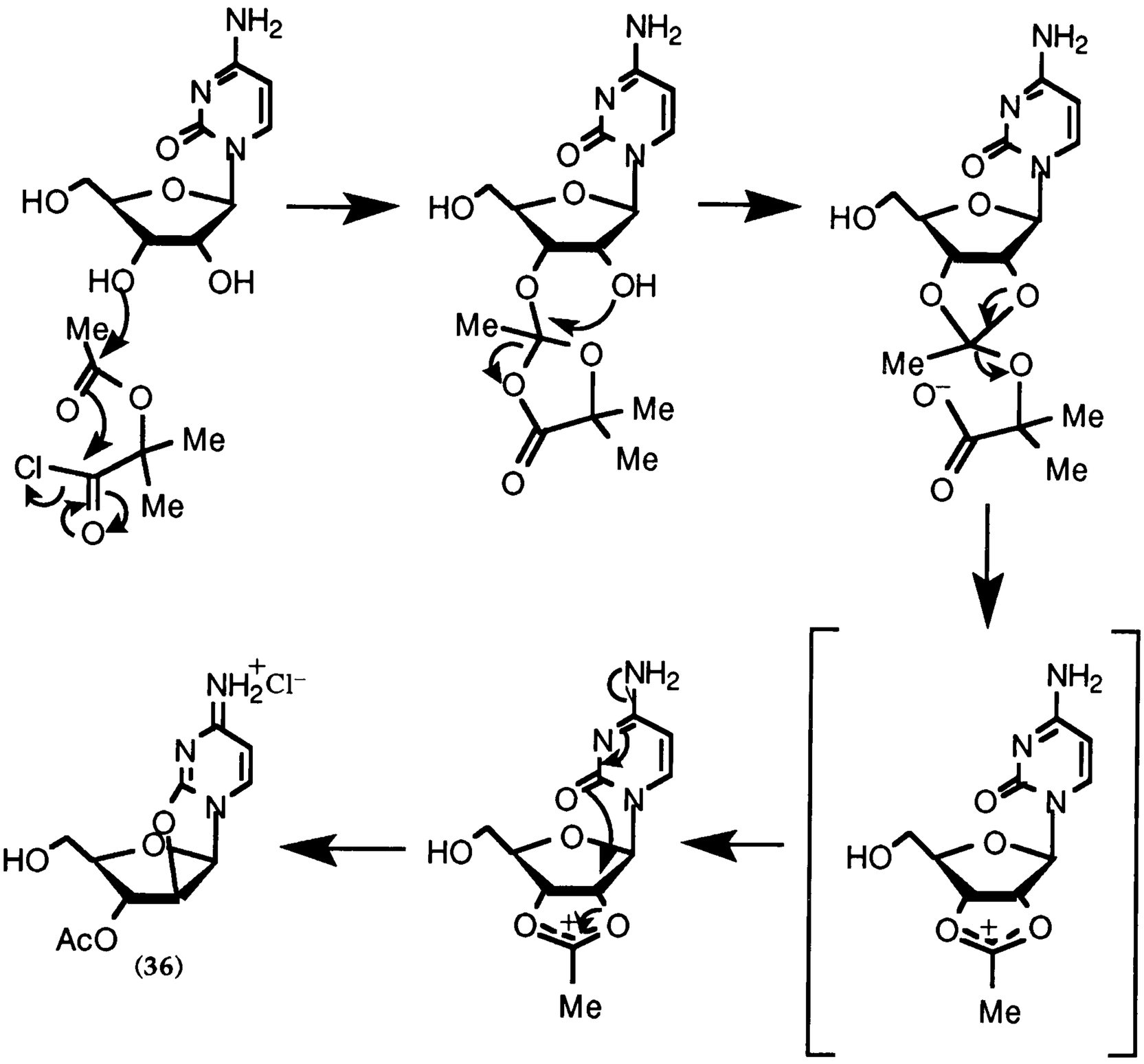
The addition of an excess of 2-acetoxyisobutyryl chloride to a suspension of cytidine in acetonitrile at 80°C afforded the crystalline 2,2′-anhydro-(3′-acetyl-β-D-*arabino*-furanosyl)cytosine hydrochloride (**36**) in 99% yield. Treatment with HCl in methanol at room temperature for 6 days removed the 3′-acetyl group to give 2,2′-anhydro-1-(β-D-*arabino*-furanosyl)cytosine hydrochloride (**37**). Fluorination with potassium fluoride in crown ether opened the cyclic nucleoside to yield the required 2′-fluoro-2′-deoxycytidine (**38**) which was converted to the uridine derivative (**39**) in 24% overall yield.



Scheme 4.20 Synthesis of 2′-deoxy-2′-fluorouridine

The reaction of 2-acetoxyisobutyryl chloride with cytidine proceeds via the conversion of the 2′,3′-cis diol into a reactive 2′,3′-acetoxonium ion, which undergoes intramolecular attack by the C_2 carbonyl group of the pyrimidine ring to initially form 2,2′-anhydro-(3′-acetyl-β-D-*arabino*-

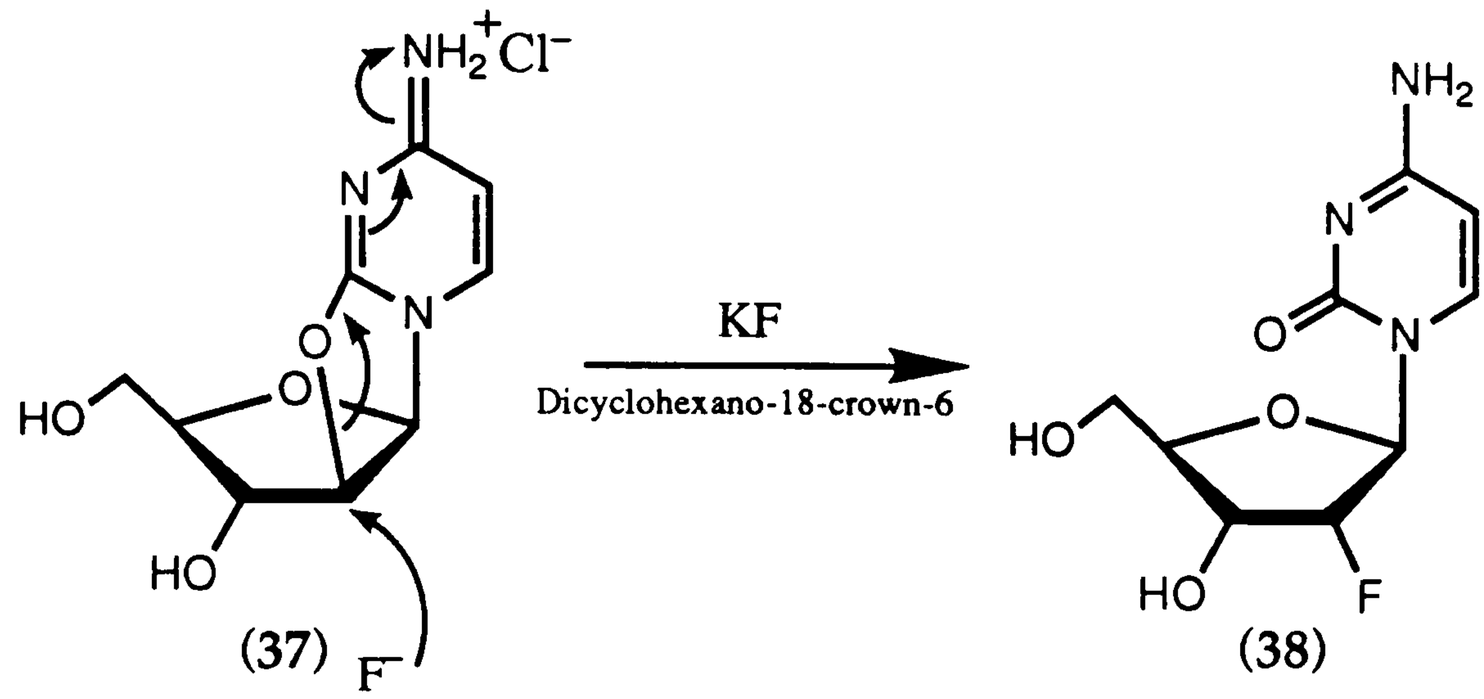
furanosyl)cytosine hydrochloride (**36**) which is then deacylated to give 2,2′-anhydro-1-(β-D-*arabino*-furanosyl)cytosine hydrochloride (**37**)[191].



Scheme 4.21 Formation of 2,2′-anhydro-(3′-acetyl-β-D-*arabino*-furanosyl)cytosine hydrochloride (**36**)

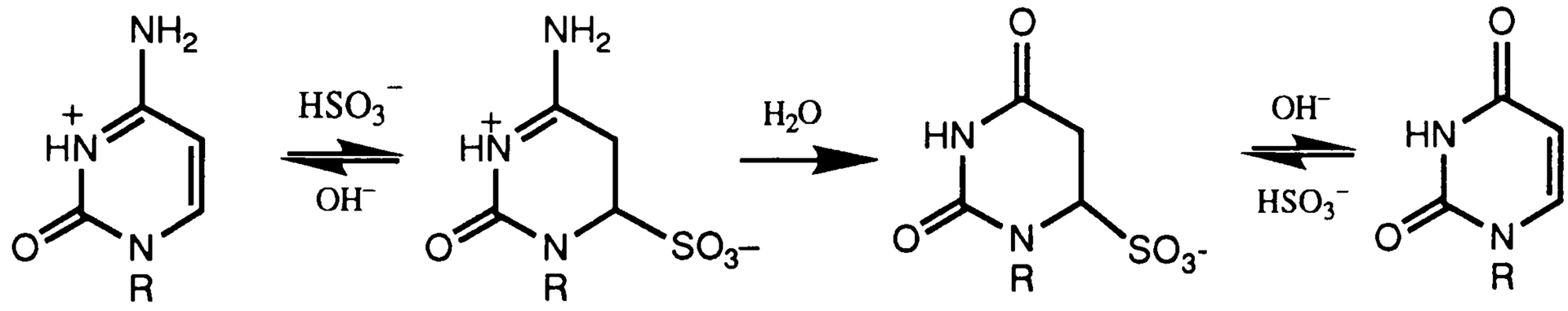
The conversion of 2,2′-anhydro-1-(β-D-*arabino*-furanosyl)cytosine hydrochloride (**37**) to 2′-fluoro-2′-deoxycytidine (**38**) was performed by activation of fluoride with crown ether under total exclusion of moisture. The crown ether forms a stable complex with the potassium ion to liberate the fluoride ion which has increased nucleophilicity. The crown ether–activated fluoride ion also has increased basicity which can lead to hydrolysis of the anhydro bond in the presence of moisture. Therefore,

only after complete elimination of traces of moisture by azeotropic distillation, with benzene from the reaction mixture, can the desired nucleophilic fluoride substitution take place[192].



Scheme 4.22 Fluorination of 2,2′-anhydro-1-(β-D-*arabino*-furanosyl)cytosine hydrochloride (**37**) with KF/crown ether

The final step is the conversion of the cytosine base to a uracil base by catalysis with bisulphite[193].



R = H, Ribose, 2-deoxyribose

Scheme 4.23 Conversion of cytosine into uracil

The sodium bisulphite adds reversibly to the 5,6-double bond of cytosine, which then deaminates upon standing in aqueous solution. If the pH is adjusted to 10 then the equilibrium is moved to the right to afford the uracil derivative.

These compounds and the others listed in Materials and Methods were used in the variable-temperature ^1H NMR conformational studies of the sugar rings.

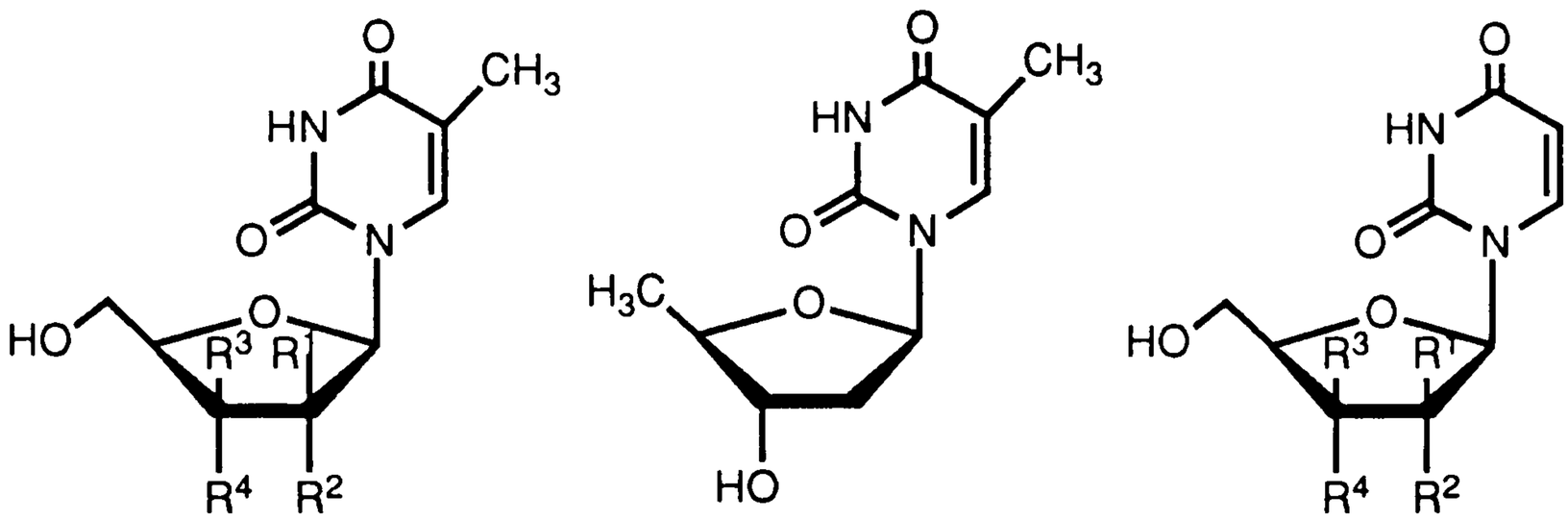
4.2.4: Conformational Studies of Nucleosides

As discussed in Chapter 2, in the synthesis of deoxyribonucleosides using N-deoxyribosyltransferases from lactobacilli to catalyse the transfer of a deoxyribosyl residue or an analogue from a donor nucleoside to an acceptor base, it has been found that 2′-deoxy-, 2′,3′-dideoxy-, and 2′,5′-dideoxynucleosides are efficient glycosyl donors but that 3′-substituted 2′,3′-dideoxynucleosides will not transfer their glycosyl residues to an acceptor base. Little is known about the active site of these enzymes, but major factors accounting for this lack of reactivity may be steric hindrance or dipolar effects that inhibit the binding of a substrate to the transferase. Another factor which may be important is the "flexibility" of the deoxyribose ring. As discussed in the introduction to this chapter, the flexibility can be considered to be made up of three components:

- rotation about the glycosyl bond;
- rotation about C–4′—C–5′;
- pseudorotation of the deoxyribosyl ring.

The pseudorotation of ten 2′- and 3′-substituted 2′,3′-dideoxynucleosides have been investigated because a substrate which adopts a "rigid", unfavourable shape may not bind to the active site of the transferase and may be inactive as a substrate. It was hoped that these studies would yield some information on the binding of these compounds to enzymes in aqueous media. The technique selected for this investigation, variable

temperature ^{1}H NMR, requires a solvent which remains liquid over as wide a temperature range as possible. Perdeuteromethanol was found to be a more suitable solvent than water, which is liquid over a comparatively narrow range of temperature, because it permitted the use of a much wider range of temperatures than is possible with water. It was found that the ^{1}H NMR spectra of solutions of the nucleosides in water and in perdeuteromethanol were very similar, suggesting that they adopt similar conformations in the two solvents and thus allowing comparisons to be made between results obtained in perdeuteromethanol and aqueous solutions.



1 $R^1 = R^2 = R^3 = H,\ R^4 = OH$
32 $R^1 = R^2 = R^3 = H,\ R^4 = F$
40 $R^1 = R^2 = R^3 = H,\ R^4 = N_3$
41 $R^1 = F,\ R^2 = R^3 = H,\ R^4 = OH$
6 $R^1 = R^2 = R^3 = R^4 = H$
35 $R^1 = R^2 = H,\ R^3 = R^4 = F$
3 $R^1 = R^2 = R^4 = H,\ R^3 = OH$

42

43 $R^1 = F,\ R^2 = R^3 = R^4 = H$
39 $R^1 = R^3 = H,\ R^2 = F,\ R^4 = OH$

Fig. 4.24 Structures of 2′- and 3′- substituted 2′,3′-dideoxynucleosides investigated by variable temperature ^{1}H NMR

From the temperature dependencies of the N and S conformer equilibria for compounds **1, 3, 6, 32, 35, 39, 40, 41, 42, 43**, the conformer populations and equilibrium constants were determined. These were used then to calculate the enthalpy and entropy differences between the N and S forms.

In theory, it would appear possible to establish the complete conformation of a sugar ring from all the vicinal proton coupling constants. Unfortunately, such an approach is not possible because the sugar ring does not possess a unique rigid structure in solution but is in a dynamic equilibrium between at least two favoured puckered conformations, a type N conformer and a type S conformer. The rapid interconversion rate between these conformers results in the observed coupling constants being averages of the coupling constants of the individual conformers. However, methods have been developed for relating coupling constants to sugar ring conformation properties and of the empirical and semi-empirical methods available, those of Altona and Sundaralingam have been the most widely used[186, 194].

4.2.4.1: Variable Temperature ^{1}H NMR Studies

The ^{1}H NMR spectra of compounds **1, 3, 6, 32, 35, 39, 40, 41, 42, 43**, were measured between 190 and 330K. In high resolution proton NMR the most widely used proton "NMR thermometers" are methanol for low temperatures (175–330K) and ethylene glycol for high temperatures (310–410K) regions. In the temperature range of this investigation the probe temperature was calculated from the chemical shift separation of the resonances of the aliphatic and hydroxyl protons of methanol[195]. At low temperature, more hydrogen bonding occurs, and the OH peak shifts downfield. Conversely as the temperature rises, the amount of hydrogen bonding diminishes, and the OH proton resonance moves upfield towards the methyl resonance.

The temperature dependence has been determined by Van Geet at 60MHz and for methanol the least squares fit gives the empirical equation:

$$T(K) = 403.0 - 0.491\ |\Delta\upsilon| - 66.2\ [10^{-2}\Delta\upsilon]^2$$

Where $\Delta\upsilon$ = chemical shift separation in Hz at 60MHz

T = temperature in Kelvin

More recent calibration of these shifts have been carried out and found to be in excellent agreement with the above equation[196]. It has been shown from work at 220MHz, that the Van Geet equation can be scaled to higher fields without any significant increase in error. Therefore, in the present study the internal temperature of the sample was determined by using the above equation with a scaling factor (60÷400 = 0.15) as the chemical shift separations were measured at 400MHz:

$$T(K) = 403.0 - 0.491\ |\Delta\upsilon| - 66.2\ [10^{-2}\Delta\upsilon]^2 \times 0.15$$

4.2.4.2: Vicinal Spin-Coupling Constants

From each spectrum the vicinal spin-coupling constants were determined and are tabulated in Appendix II. These vicinal spin-coupling constants[186] were used to calculate the puckering equilibrium of the deoxyribose rings (Table 4.1), using the formula:

$$\%S = 100 \times J_{1'2'}/(J_{1'2'} + J_{3'4'}).$$

where %S = Type S conformer population

$J_{x'y'}$ = the coupling constants relating to the structure:

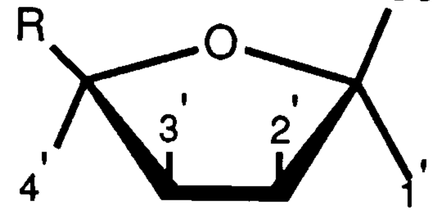


Fig. 4.25 Coupling constants in the deoxyribose ring

The equilibrium constant for each compound (Table 4.1) was calculated also by the use of these vicinal spin-coupling constants using the formula:

$$K_{eq} = J_{1'2'}/J_{3'4'} = X_S/X_N$$

where K_{eq} = equilibrium constant

$J_{x'y'}$ = coupling constants relating to the above structure

X_S = mole fraction of conformer type S

X_N = mole fraction of conformer type N

The coupling constants due to protons in the glycosyl moieties of the nucleosides must obey inter-relationships governed by the laws of pseudorotation and, hence, the coupling constants cannot assume random values that are independent of each other. This situation allowed predictions to be made for values of $J_{H,H}$ when fluorine atoms replaced hydrogen atoms in the glycosyl ring and only $J_{H,F}$ could be measured[197]. When measuring the coupling constants for fluorinated deoxyribose moieties, it was assumed, where necessary, that the corresponding $J_{H,H} = 1/2(J_{H,F})$. This adjustment of coupling constants was not required for the ΔH and ΔS calculations, but such a correction factor was required to

ΔH and ΔS calculations, but such a correction factor was required to estimate a value for the percentage of N- and S-character in the conformations adopted by fluorinated nucleosides.

The puckering equilibrium values and the equilibrium constants, along with the vicinal spin-coupling constants used to calculate them, have been tabulated (Table 4.1):

Table 4.1 Variation in coupling constants in the deoxyribosyl ring of nucleosides with temperature

Compound	Temp/K	J/Hz		K_{eq}	%S
		$H_{1',2'}$	$H_{3',4'}$		
1	209	6.91[a]	2.70	2.56	74
	237	6.80[a]	3.00	2.27	69
	298	6.80[a]	3.35	2.03	67
	315	6.78[a]	3.46	1.96	67
32	208	9.69	0	[b]	>99
	259	9.60	0	[b]	>99
	278	9.37	0	[b]	>99
	297	9.25	0	[b]	>99
	315	9.13	0	[b]	>99
40	208	6.33[d]	4.49	1.41	59
	233	6.36[d]	4.70	1.35	58
	259	6.38[d]	4.79	1.33	57
	279	6.40[d]	4.84	1.32	57
	302	6.43[d]	4.85	1.33	57
	310	6.43[d]	4.80	1.34	57
42	234	6.47	3.90	1.66	62
	280	6.68	4.09	1.63	62
	296	6.69	4.16	1.61	62
	321	6.70	4.27	1.57	61
41	208	16.76[c]	4.82	1.74	64
	259	16.85[c]	4.77	1.77	64
	279	16.94[c]	4.84	1.75	64
	300	17.03[c]	4.85	1.76	64
	319	17.09[c]	4.84	1.77	64

Compound	Temp/K	J/Hz		K_{eq}	%S
		$H_{1',2'}$	$H_{3',4'}$		
43	208	18.69[c]	5.06	1.85	65
	234	18.22[c]	5.22	1.75	64
	259	17.85[c]	5.41	1.65	62
	279	17.62[c]	5.54	1.59	61
	297	17.42[c]	5.67	1.54	61
	320	17.35[c]	5.70	1.52	60
6	209	2.30	8.82	0.26	21
	234	2.73	8.49	0.32	24
	260	3.12	8.74	0.36	26
	276	3.26	8.70	0.37	27
	297	3.49	8.68	0.48	32
	320	3.59	7.29[a]	0.49	33
35	206	9.01	3.72[d]	4.84	83
	215	8.94	4.20[d]	4.26	81
	232	8.75	5.19[d]	3.37	77
	252	8.56	6.67[d]	2.56	72
	269	8.39	6.62[d]	2.53	72
	296	8.13	7.37[d]	2.21	69
	317	7.54	7.93[d]	1.90	66
39	211	<1.0	8.27	0.12	11
	235	1.19	8.09	0.15	13
	260	1.85	7.33	0.25	20
	278	2.15	7.27	0.29	23
	297	2.23	7.26	0.31	24
	307	2.34	7.23	0.32	24
3	198	1.64	3.02	0.54	35
	214	1.86	2.95	0.63	39
	231	1.99	3.04	0.66	40
	249	2.17	3.12	0.70	41
	269	2.31	3.12	0.74	43
	296	2.52	3.12	0.81	45

[a]Average value determined from unresolved peak signals, [b]Very large as $J_{3',4'}$ = 0Hz, [c]$J_{1',F}$, [d]$J_{4',F}$.

For each compound, the differences in enthalpy and entropy between the favoured conformations adopted over the range of temperatures studied were calculated from the temperature dependence of the equilibrium constants by using a weighted least-squares fit for the van't Hoff equation:

$$-\log K_{eq} = \Delta H/RT - \Delta S/R$$

A graph was plotted of 1/T in Kelvin versus ln K_{eq} and from this the intercept gave the value of ΔS and the gradient gave the value of ΔH which have been tabulated (Table 4.2):

Compound	Enthalpy difference[a] (kcal/mol)	Entropy difference[a] (e.u.)
1	–0.43 (0.08)	–0.50 (0.35)
32	0.0 (0.0)	+9.13 (0.0)
40	–0.11 (0.02)	+0.16 (0.08)
42	–0.13 (0.01)	+0.495 (0.04)
41	+0.02 (0.01)	+1.18 (0.02)
43	–0.25 (0.01)	+0.029 (0.02)
6	+0.82 (0.14)	+1.17 (0.49)
35	–1.13 (0.08)	–2.38 (0.34)
39	+1.29 (0.19)	+2.04 (0.70)
3	+0.43 (0.03)	+1.03 (0.13)

[a]Standard deviations are given in parentheses.

Table 4.2 Enthalpy and entropy differences between N and S conformations of nucleosides

These structures were then modelled on a Silicon Graphics workstation using QUANTA, which gave good agreement with the calculated conformations.

The main interests of this study lay in determining the "flexibility" and preferred conformational type of each sugar ring in the 2′- and 3′-substituted-2′,3′-dideoxynucleosides. From the data it can be seen that the ^{1}H NMR spectra of 3′-fluoro-3′-deoxythymidine (**32**) in perdeuteromethanol do not vary significantly over the temperature range 208–315K (Table 4.1 and Fig. 4.26). The calculated difference in enthalpy between the N– and S–conformations of (**32**) over this temperature range is zero. On the other hand, there is a large entropy difference (+9.1e.u.) between the N– and S–conformations over this temperature range (Table 4.2). This finding suggests that the conformation of the deoxyribosyl ring in (**32**) has >99% S–character, implying that (**32**) adopts a "rigid" shape with an extreme S–conformation. When Van Roey and Schinazi[198] analysed the crystal structure of 3′-fluoro-3′-deoxythymidine (**32**), they found that the conformation has a pseudorotation angle, P, of nearly 180^o which corresponds to a nearly perfect C2′-endo/C3′-exo twist and agrees with our findings of a 99% S conformation.

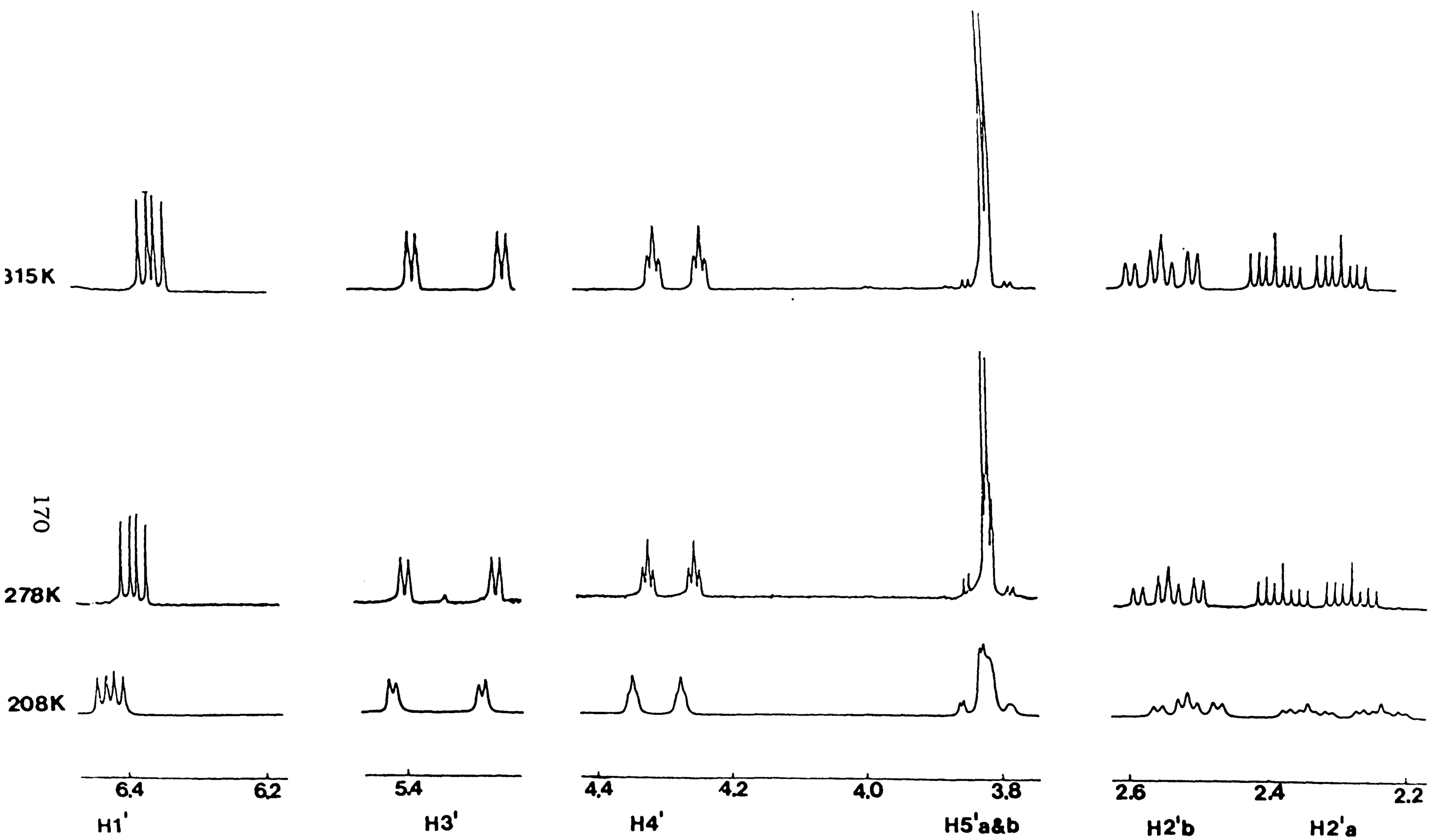
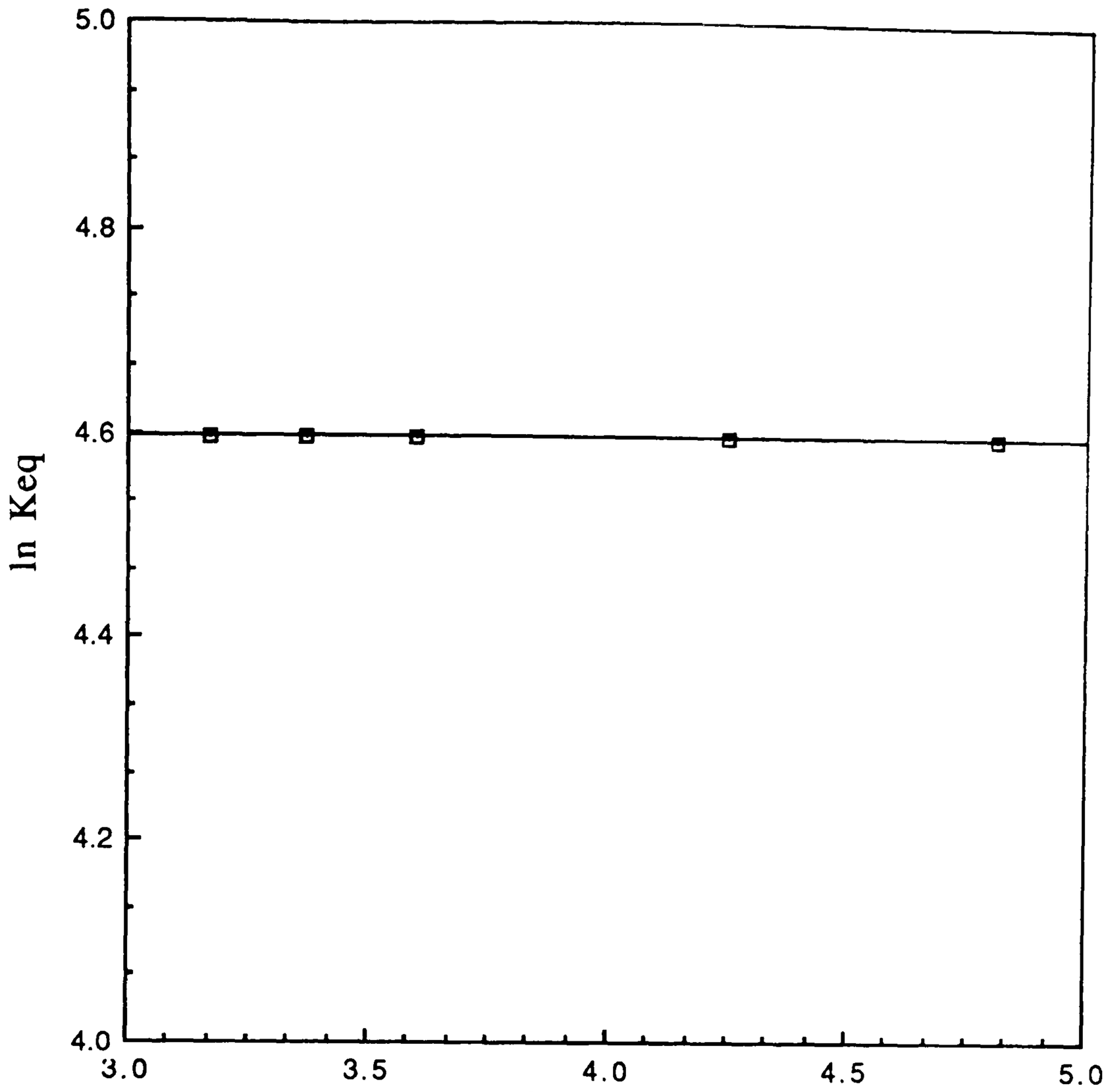


Fig. 4.26 ^{1}H NMR spectra of 3′-fluoro-3′-deoxythymidine (32) run at 400MHz in CD_3OD measured at 208, 278, 315K.



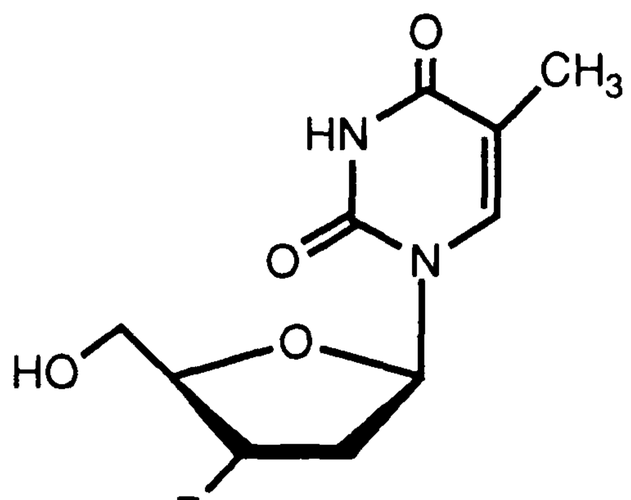


Fig. 4.27 The variation of the equilibrium constant with temperature for 3′-fluoro-3′-deoxythymidine (**32**)

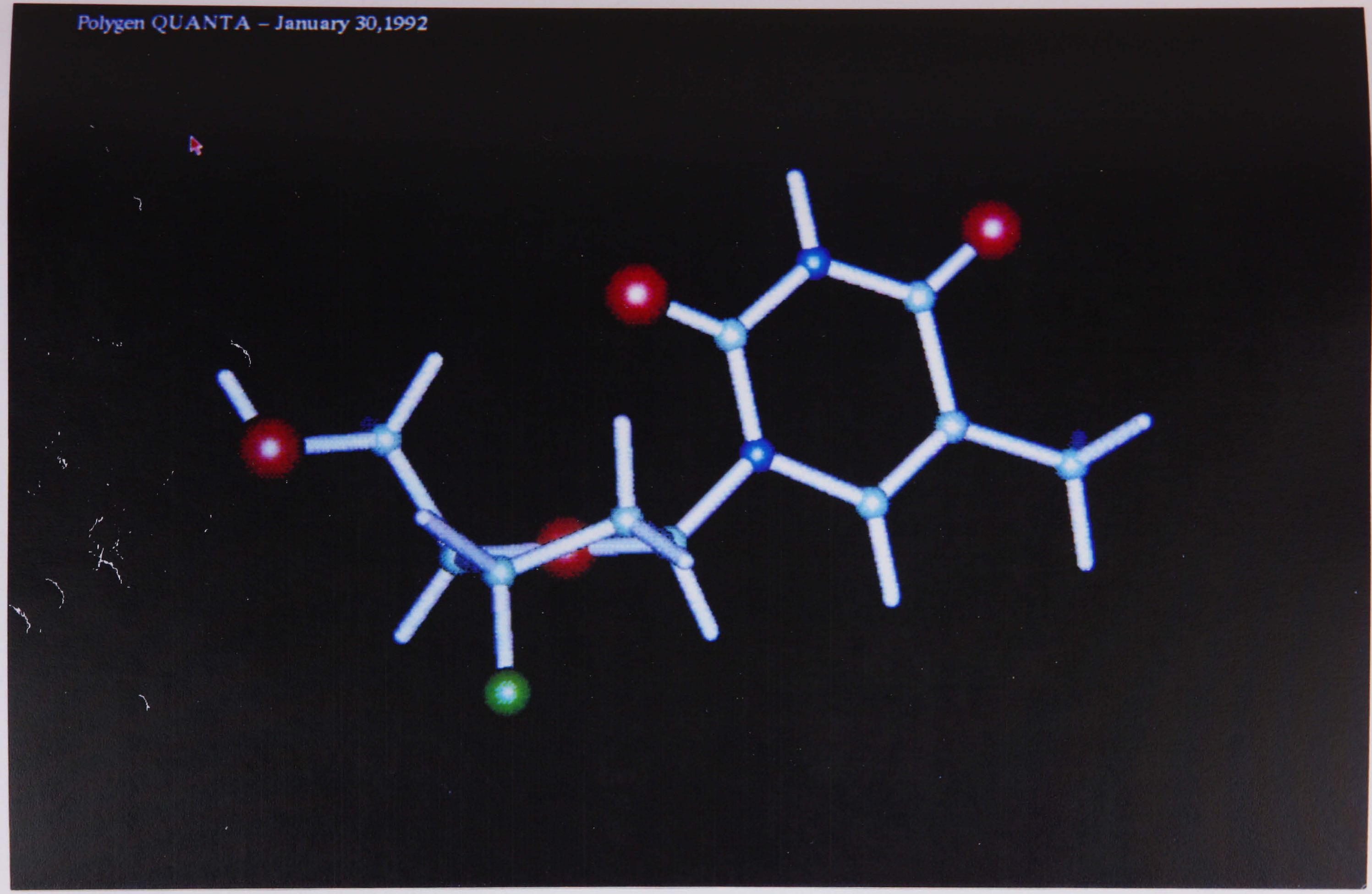


Fig. 4.28 Structure of 3′-fluoro-3′-deoxythymidine (**32**) produced by molecular modelling

The NMR spectra of 3′-azido-3′-deoxythymidine (**40**), 5′-deoxythymidine (**42**), and 2′-deoxy-2′-fluoro-5-methyl-arabinosyluracil (FMAU, **41**) also changed little over this temperature range. Calculations indicate that there are small differences in enthalpy between the N– and S–conformations of the three compounds over this range of temperatures. However, the magnitude of the entropy differences between the N– and S–conformations increase with increasing S–character (**40**, +0.16 e.u., 59% S–character; **42**, +0.495 e.u., 62% S–character; **41**, +1.18 e.u., 64% S–character). The small entropy values for 3′-azido-3′-deoxythymidine (**40**) and 5′-deoxythymidine (**42**) suggest that these compounds assume a favoured conformation which is unaffected by changes in temperature and is therefore "rigid". On the other hand, for 2′-deoxy-2′-fluoro-5-methyl-arabinosyluracil (FMAU, **41**), there is a comparatively large difference in entropy (1.16 e.u.) between the N– and S–conformations, which could make the deoxyribosyl ring relatively "inflexible", causing the molecule to have a nearly constant percentage (65%) of S–character over the temperature range studied.

The NMR spectra of the other nucleosides studied changed with change in temperature, indicating that the sugar rings show some conformational mobility under these conditions. The enthalpy differences for 3′-deoxythymidine (**6**), 2′-fluoro-2′-deoxyuridine (**39**), and 1-(2′-deoxy-β-D-*erythro*-pentofuranosyl)thymine (**3**) are +0.82, +1.29, +0.43 kcal/mol respectively, indicating that, for these compounds, the N–conformation is more stable than the S–conformation. As the temperature is lowered, so the favoured N–conformations become more prevalent, indicating that the deoxyribosyl rings are "flexible".

In contrast, for 3′,3′-difluoro-3′-deoxythymidine (**35**), there was a large negative (-1.13 kcal/mol) difference in enthalpy between the two forms, showing that the S–conformation is significantly more stable (Table 4.2 and Fig. 4.29). As the temperature is reduced, the flexibilty of the deoxyribosyl ring is again shown by the increase in S–character from 69% at 296K to 83% at 206K (Table 4.1).

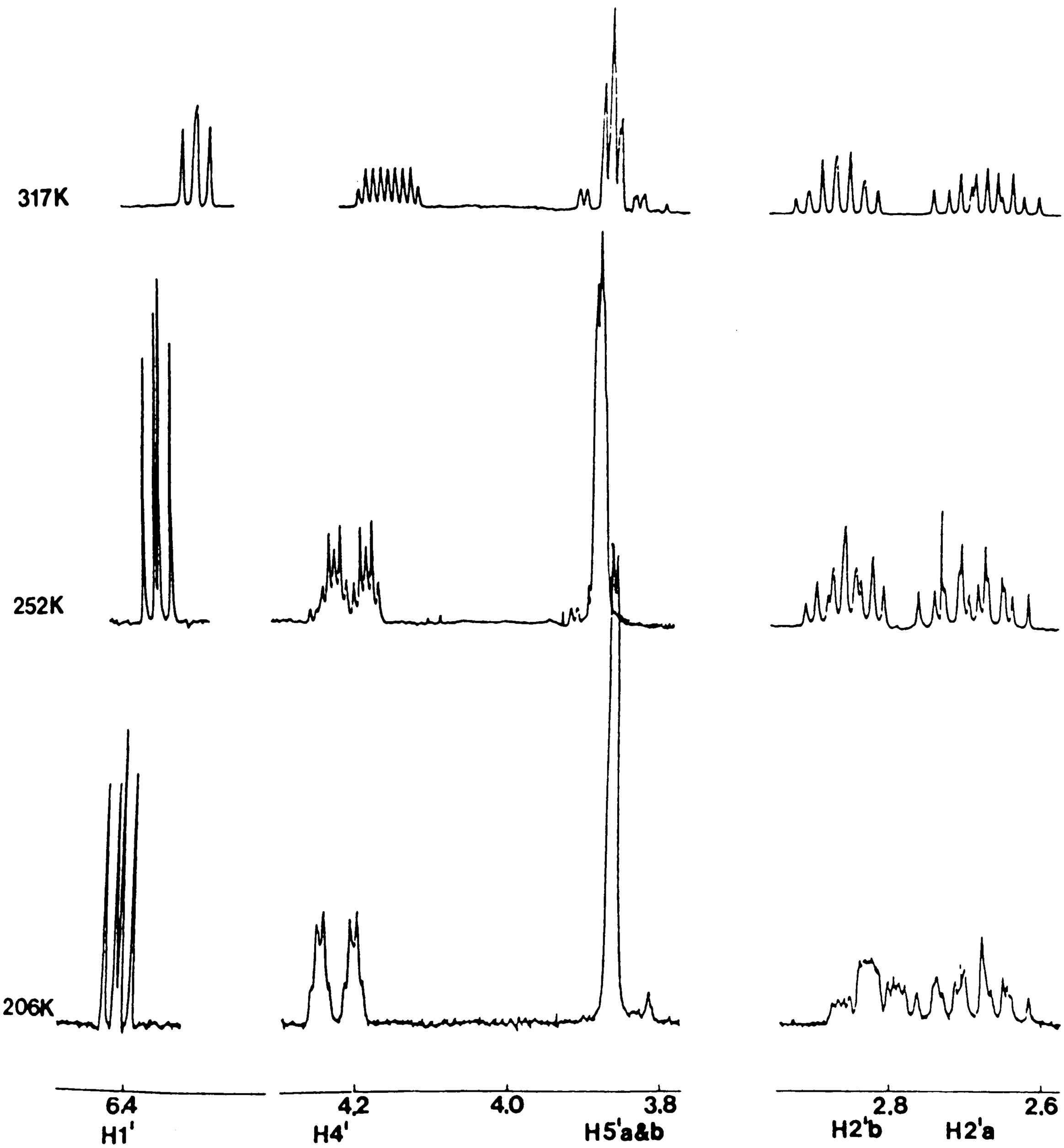
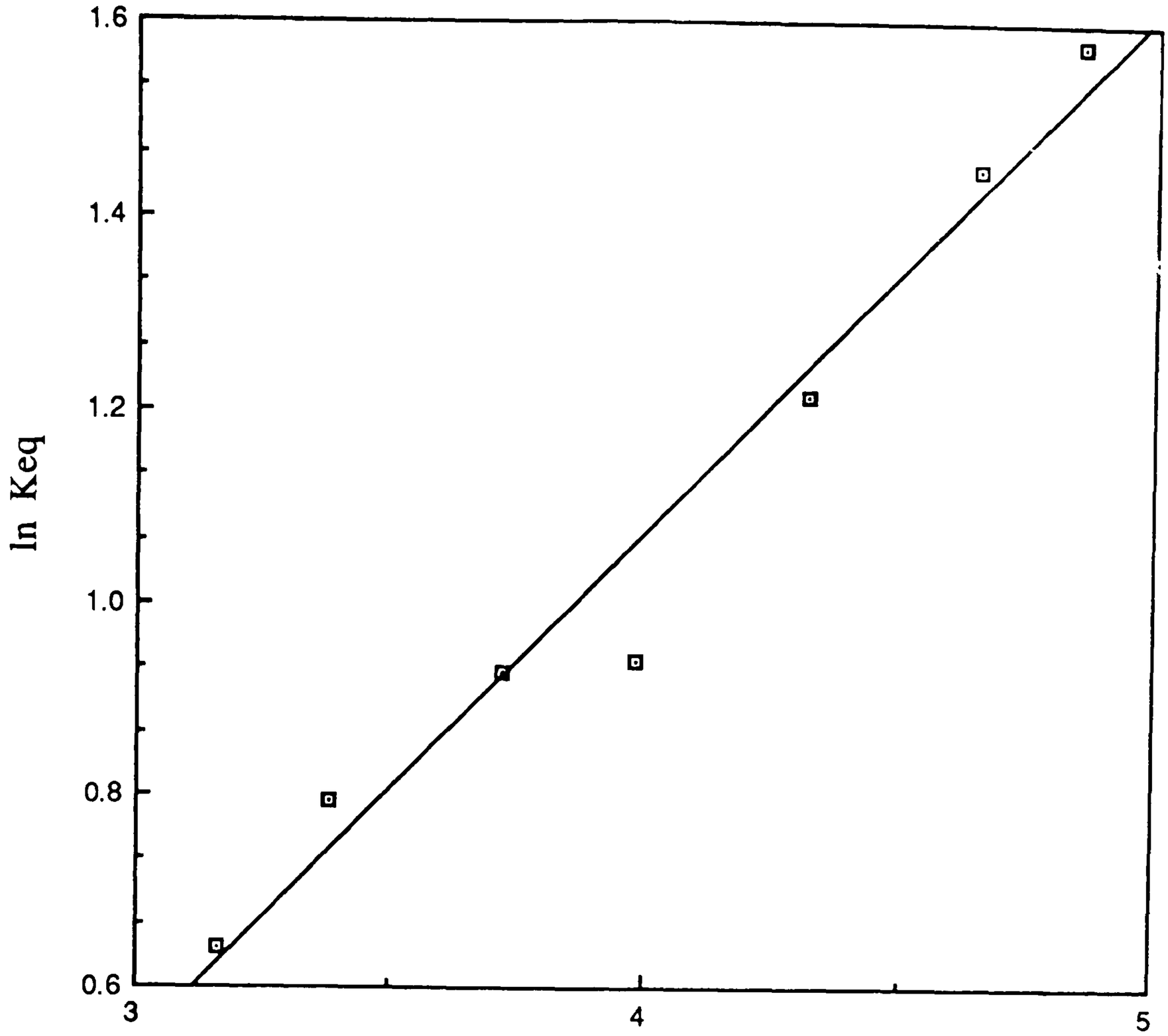


Fig. 4.29 ^{1}H NMR spectra of 3′,3′-difluoro-3′-deoxythymidine (**35**) run at 400MHz in CD_3OD measured at 206, 252, 317K



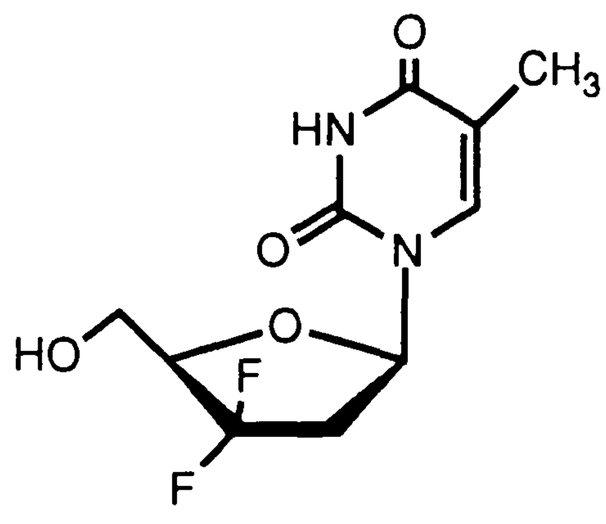


Fig. 4.30 The variation of the equilibrium constant with temperature for 3′,3′-difluoro-3′-deoxythymidine (**35**)

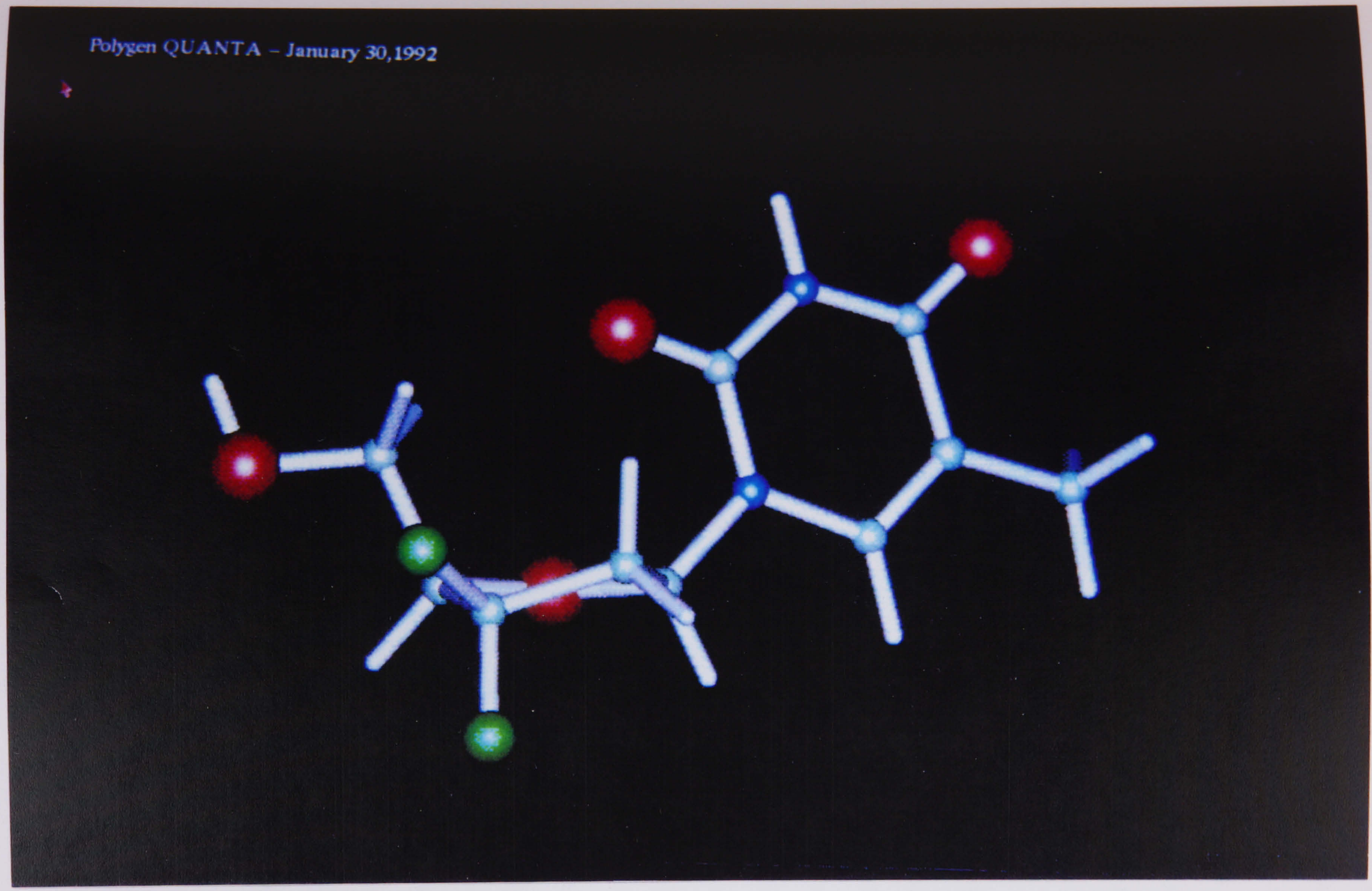


Fig. 4.31 Structure of 3′,3′-difluoro-3′-deoxythymidine (35) produced by molecular modelling

The remaining compounds, thymidine (**1**) and 1-(2′,3′-dideoxy-2-fluoro-β-D-*threo*-pentofuranosyl)uracil (**43**) show an increased percentage of the favoured S–conformation at low temperatures. The relatively small enthalpy and entropy values for these compounds suggest that the sugar rings are reasonably "flexible". The conformations which were elucidated for all these compounds are in good agreement with those published in the literature[177, 198-200].

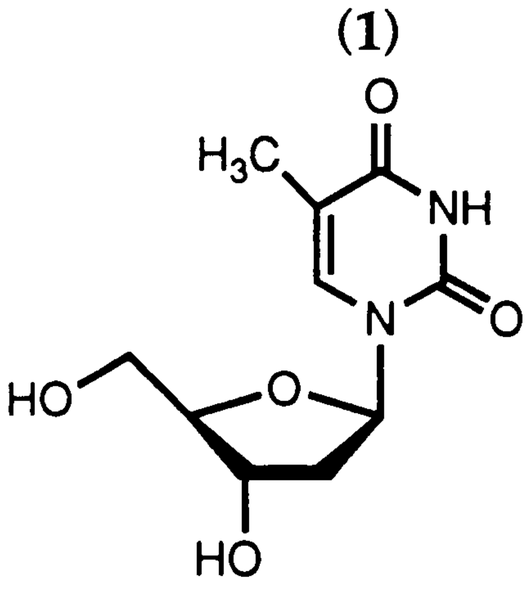
The position and orientation of substituents have a definite and predictable influence on the conformation of the deoxyribose ring. Atoms or groups on the same side of the sugar ring as C–5′ are designated *endo* and those on the opposite side of the ring to C–5′ are *exo*. Those nucleoside analogues in which the 3′-substituent is *exo* favour the S–conformation, whereas those analogues with the 3′-substituent *endo* adopt N–conformations. These predictions can also be extended to the 2′-position. An *exo* 2′-substituent appears to favour the adoption of the N–conformation, while an *endo* 2′-substituent favours the adoption of the S–conformation. In 2′,3′-disubstituted compounds, the influence of the substituents is additive. For example, 2′-fluoro *endo* and 3′-hydroxy *exo* substitution in 2′-deoxy-2′-fluoro-5-methyl-arabinosyluracil (FMAU, **41**) enhances the amount of S–character in the conformation and renders the sugar ring almost "inflexible". However, as can be seen in 2′-fluoro-2′-deoxyuridine (**39**), the influence of the 2′-fluoro *exo* substitution over-rides the effect of the 3′-hydroxyl *exo* substitution. The resulting nucleoside has the N–conformation and the sugar ring is still "flexible". This observation, that the most polar substituent pulls the pucker to its side, has been investigated and discussed in several publications[201, 202].

As mentioned before, although the fluorine atom is only slightly larger than the proton, it is considerably more electronegative. It appears, therefore, that the sugar conformation is essentially influenced by the polarity of the substituents. ^{1}H and ^{13}C NMR studies, which have been done to determine the relationship between electronegativity of 2′-substituents and conformation of the sugar moiety of adenosine, have found a linear correlation. When studying 2′-substituted 2′-deoxyadenosine it was found that there is an increase in the contribution of the N–conformer with the increasing electronegativity of the 2′-substituent in the order F>N_3>Cl>Br>I. This pattern also corresponds to the atomic radii of these groups so steric hindrance may be occurring. However, the compounds substituted at C–2′ by OH, OMe, N_3 and NH_2, which are bulkier than H, have predominately more of the N–conformer than the H–substituted compounds, thus inferring that there is only a minor steric effect.

These results show the dominant influence of the polarity of the substituent on the conformation of the sugar ring (Pauling's electronegativites: F = 4.0, OH = 3.5, N_3 = 3.0, Cl = 3.0, NH_2 = 3.0, H = 2.1). For the substituents at C–2′ and C–3′ the most electronegative substituents pulls the pucker towards its side. This is shown by 2′-deoxyadenosine having the highest preference for the S–form, while 2′-fluoro-2′-deoxyadenosine has the highest preference for the N–form. Hence, it appears that polar effects of the C–X bond rather than steric considerations are mainly responsible for the adoption of the preferred conformation. This preference for the electronegative substituents to be approximately *gauche* to the ring O4′ rather than trans is known as the *gauche* effect[163]. However, the effects of hydrogen-bonding with the solvent must not be discounted as the binding will be different for every substituent.

4.2.5: Summary

Those compounds which are active glycosyl donors with N-deoxyribosyltransferases from lactobacilli, thymidine (**1**), 5′-deoxythymidine (**42**), and 3′-deoxythymidine (**6**), appear to have relatively flexible sugar rings. This property may allow them to adopt the "correct" conformation at the active site of the transferase without undue input of energy. Steric and dipolar constraints may become important in the case of 3′-fluoro-3′-deoxythymidine (**32**) and AZT (**40**), making them inactive substrates for the transferases. Previous investigations found that although the sugar moiety of 3′-fluoro-3′-deoxythymidine (**32**) was not able to be transferred, some degree of hydrolysis was observed. This may indicate that the compound actually binds to the enzyme but the lack of any transfer may be due to the sugar binding weakly and the extreme conformation preventing further reaction with the acceptor base[114]. Unfortunately, further theoretical investigations into the nucleoside-enzyme interactions at the active site seem superfluous now that the structure of the enzyme has been investigated. Sadly, only preliminary findings have been published[116] and no crystal structure or other data are available.

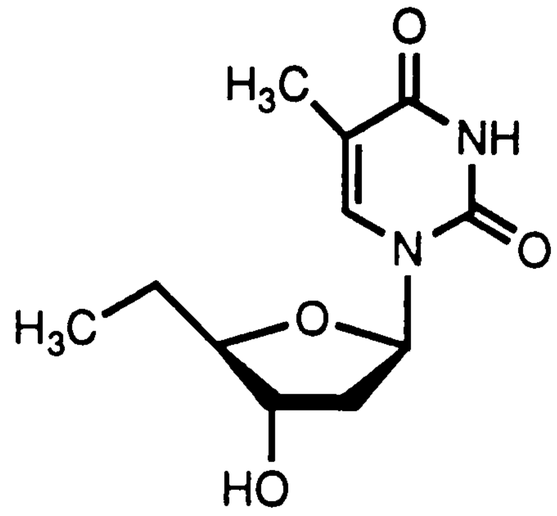


(1)

Thymidine

67% S at 294K

Flexible

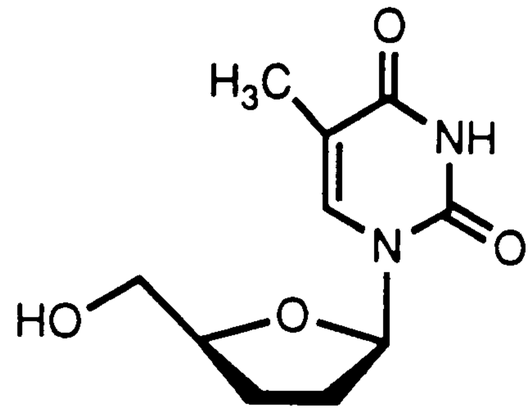


(42)

5'-deoxythymidine

62% S at 294K

Flexible

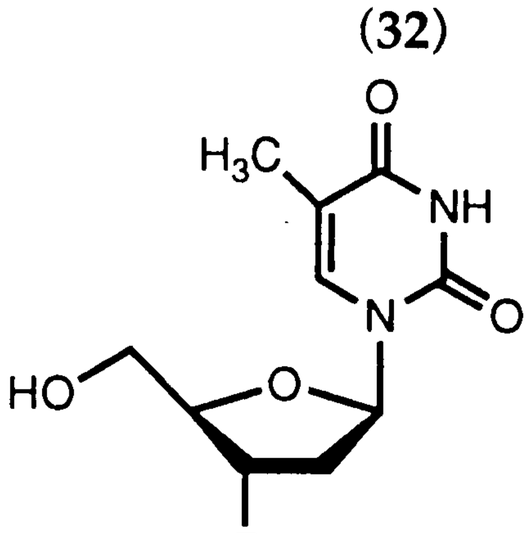


(6)

3'-deoxythymidine

32% S at 294K

Flexible



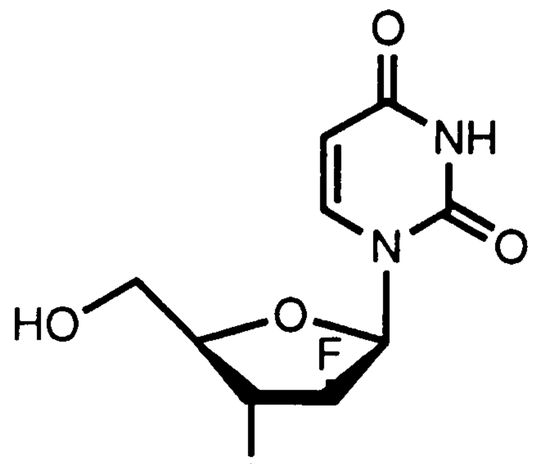
(32)

F

3'fluoro--3'-deoxythymidine

>99% S at 294K

Rigid



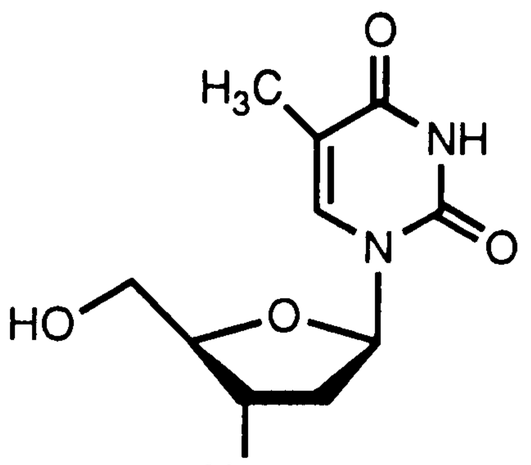
(41)

HO

FMAU

64% S at 294K

Rigid



(40)

N_3

AZT

32% S at 294K

Rigid

Fig. 4.32 Correlation of sugar conformation with antiviral activity

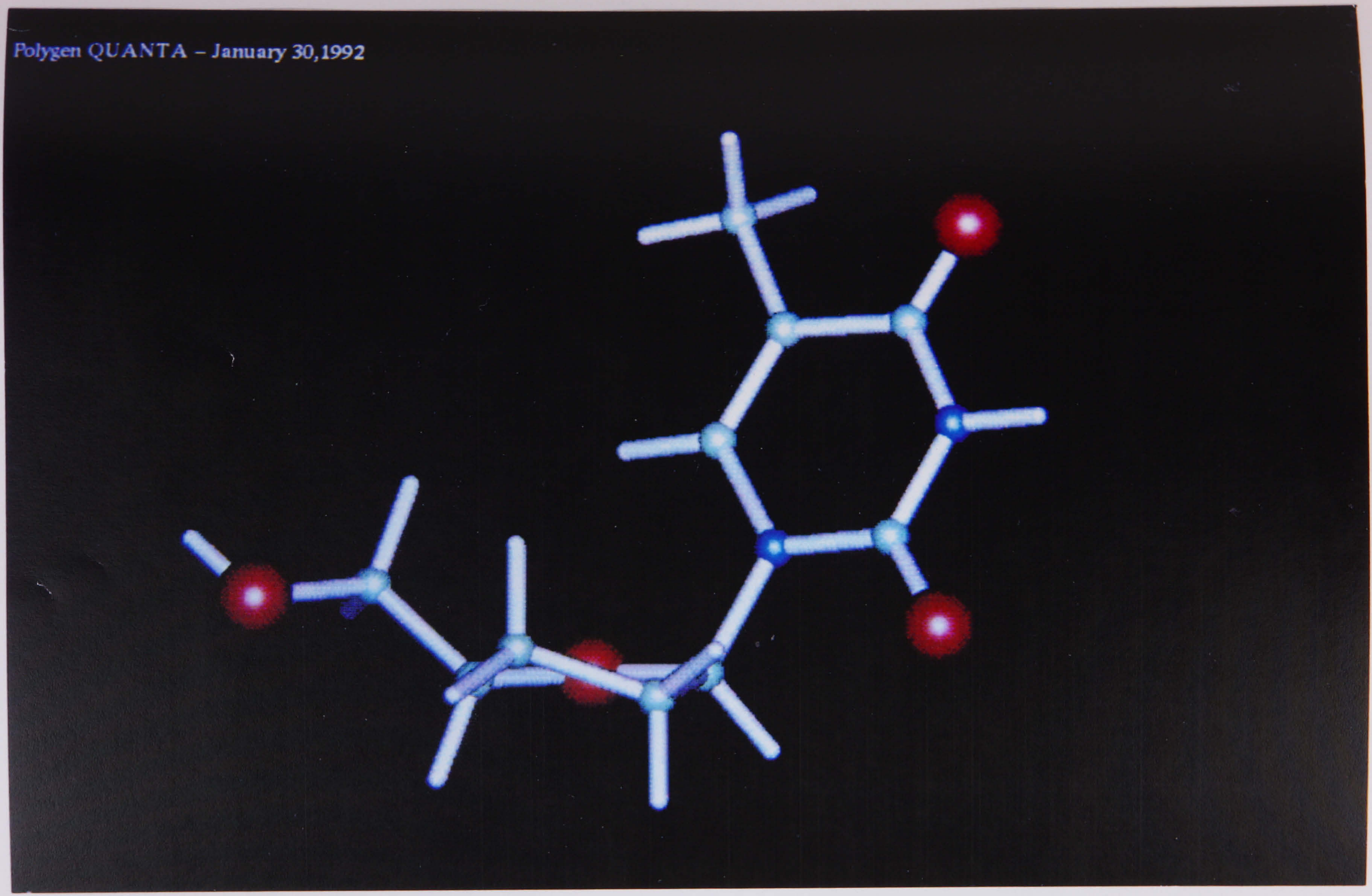


Fig. 4.33 Structure of 3′-deoxythymidine (6) produced by molecular modelling

The correlation of the shape of the sugar ring in a nucleoside in solution with antiviral activity is much more complex as several enzymic steps are involved before the nucleoside inhibits viral replication. That aside, it is of interest that three of the nucleosides in our study, 3′-fluoro-3′-deoxythymidine (**32**), AZT (**40**), and FMAU (**41**), which have "rigid" ring conformations with a predominance of S-character present at 294K, are all active antiviral agents. However, it must be emphasised that the suggestion that an S–conformation is desirable for the anti–HIV activity of nucleosides[198] is not supported by the results found for 2′,3′-dideoxynucleosides. In this work, 2′,3′-dideoxythymidine was found to have 68% N–character at 297K and this preference for the N–conformation has been shown for the other natural 2′,3′-dideoxynucleosides[199].

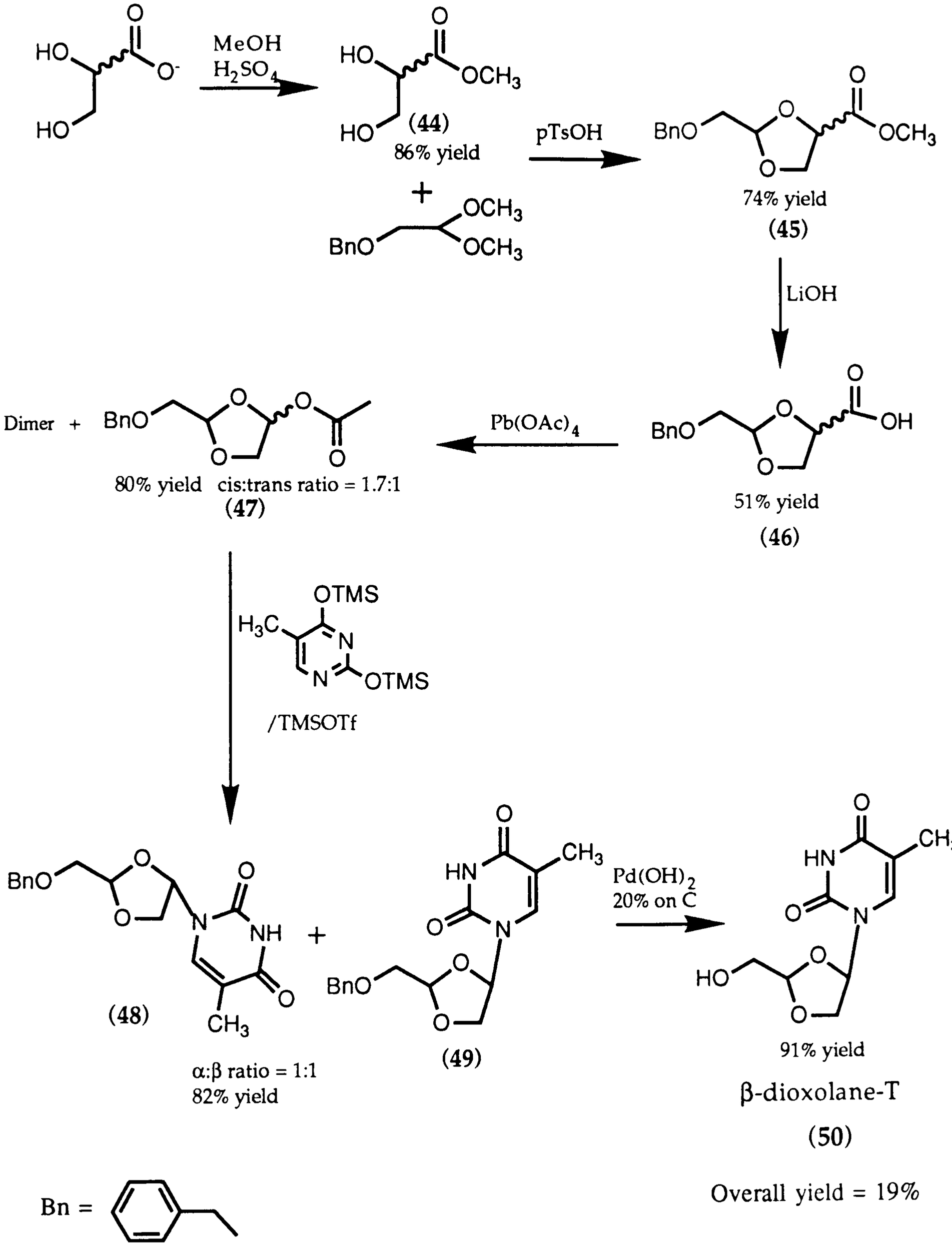
4.2.6: Synthesis of (±)-1-[(2β′,4β′)-2′-(hydroxymethyl)-4′-dioxolanyl] thymine. [(±)-Dioxolane–T] (50)[184]

Dioxolane–T (**50**) has been synthesised, in the literature, by two routes which both involve a key intermediate, (±)-4-acetoxydioxolane-2-substituted dioxolane (**47**), which is then condensed with a heterocyclic base and deprotected to afford the required nucleoside:

- the method of Kim *et al*[203] synthesised the intermediate in nine steps from 1,6-anhydro-D-mannose;

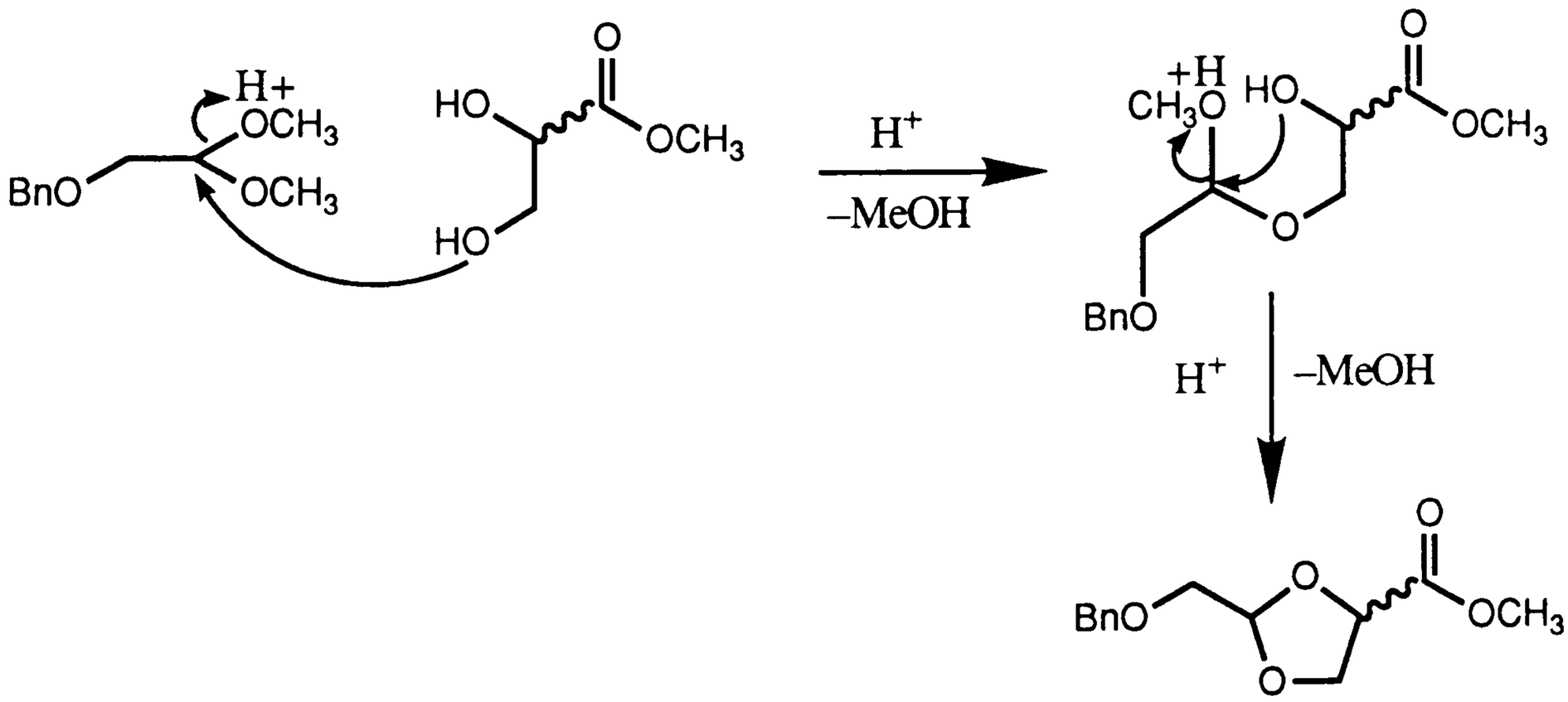
- the method of Norbeck *et al*[184] synthesised the intermediate in four steps which was the method employed in this study.

The following synthetic scheme was developed from the note by Norbeck *et al*[184] which did not give full experimental details:



Scheme 4.34 Total synthesis of (±)-Dioxolane-T[184] (50)

Fischer esterification of (±)-calcium glycerate dihydrate gave (±)-methyl glycerate (**44**) which was condensed with benzyloxyacetaldehyde dimethyl acetal to give the basic dioxolane structure (**45**) in 74% yield as a racemic, 1:1 mixture of diastereisomers. Saponification of the dioxolane methyl esters (**45**) followed by acidification afforded the carboxylic acids (**46**) as an oil. Treatment of the carboxylic acids with 1.1 equivalents of pyridine and 1.3 equivalents of lead tetraacetate in dry acetonitrile effected the oxidative decarboxylation to yield the acetates (**47**) in a 1.7:1 mixture of cis and trans isomers as the intermediate for the condensation reactions with heterocyclic bases. There was also a very polar product which was a dimer resulting from self-coupling. The acetates (**47**) were then condensed with trimethylsilylthymine[61] to yield a 1:1 mixture of diastereisomers of the dioxolanes (**48** and **49**) which were separated on silica gel. The required β-dioxolane-T (**50**) was obtained in 19% overall yield by hydrogenolysis of the cis isomer.



Scheme 4.35 Condensation of benzyloxyacetaldehyde dimethyl acetal and (±)-methyl glycerate (**44**)

The initial condensation reaction to form the basic dioxolane structure produced methanol as a byproduct and in order for the reaction to go to completion the methanol was distilled off after one hour of the reaction, forcing the equilibrium towards products in 74% yield. Saponification and acidification of the esters (**45**) afforded the carboxylic acids (**46**). The oxidative decarboxylation of the acids (**46**) to the acetates (**47**) was accomplished using lead tetraacetate and pyridine. The mechanism is generally accepted to be of the free-radical type.

Thymine was silylated using excess hexamethyldisilazane which was evaporated and the residue used immediately in the condensation reaction with the (±)-dioloxane esters (**47**) according to the general method of Vorbrüggen[61] to afford a 1:1 diastereisomeric mixture of 5′-protected (±)-dioxolane–T (**48** and **49**) which, after separation, were hydrogenolysed to yield (±)-dioxolane–T (**50**).

4.2.6.1: Variable Temperature ^{1}H NMR Studies of (±)-Dioxolane–T (50)

In order for a nucleoside to be an effective glycosyl donor in transfer reactions using N-deoxyribosyltransferase enzymes we believe that the sugar moiety of the nucleoside must be "flexible" with a conformation near to 50:50 N:S and so extremes of conformation and relatively "rigid" structures are not glycosyl donors. Norbeck *et al*[184] and Kim *et al*[203] reported that (±)-dioxolane–T (**50**) adopts an N-conformation so it was of interest to see the degree of flexiblity within the sugar moiety of this nucleoside. As there was no $H_{3'}$ proton in the structure, the $J_{H3'-H4'}$ could not be measured and therefore the conformation was calculated using the approximation $\%S = 10J_{H1'-H2'a}$[186].

TEMP/^{o}K	J /Hz		K_{eq}	%N
	$H_{1',2'a}$	$H_{1',2'b}$		
323	1.86	5.63	0.23	81
313	1.78	5.61	0.22	82
303	1.71	5.66	0.20	83
294	1.56	5.62	0.19	84
273	1.24	5.60	0.14	88
263	1.09	5.56	0.12	89

Table 4.3 Variation in coupling constants in the sugar moiety of (±)-Dioxolane–T (**50**) with temperature

The results indicated that the N-conformation adopted by the sugar ring in (±)-dioxolane–T (**50**) was an extreme N-twist. The preference for the N–conformer was shown by the increase in the %N with the decrease in temperature. The spectrum was measured at lower temperatures than indicated in the table but the resolution was too poor to yield any useful information. From these results it was unlikely that (±)-dioxolane–T (**50**) would act as a glycosyl donor in the transfer reactions but several test reactions were set-up using adenine as the acceptor base and the reactions were followed by reverse phase HPLC for one week; as expected, no transfer was observed. For completeness (±)-dioxolane–T was also investigated as a donor nucleoside in transfer reactions which use nucleoside phosphorylases as the biocatalysts; again no reaction was observed.

In summary, the synthesis of β-dioxolane-T (**50**) was successful but this nucleoside adopted an extreme N–conformation which was not accepted as a substrate by N-deoxyribosyltransferase from *Lactobacillus leichmannii*.

4.3: MATERIALS AND METHODS

4.3.1: Nucleosides

Thymidine (**1**), 3′-azido-3′-deoxythymidine (**40**) and 5′-deoxythymidine (**42**) were obtained from Sigma Chemical Co., 2′-deoxy-2′-fluoro-5-methyl-arabinosyluracil (FMAU) (**41**) was a gift from Dr. J. J. Fox (Sloan-Kettering Institute for Cancer Research, New York), 1-(2′,3′-dideoxy-2′-fluoro-β-D-*threo*-pentofuranosyl)uracil (**43**) was a gift from Dr. J. A. Martin (Roche Products, Welwyn, U.K.). 1-(2′-deoxy-β-D-*erythro*-pentofuranosyl)thymine (**3**) and 3′-deoxythymidine (**6**) were synthesised as described in Chapter 2 and the other nucleosides (**32, 35, 39**) were prepared as described in the Experimental section.

4.3.2: Variable Temperature ^{1}H NMR

The ^{1}H NMR spectra were measured on a Bruker WH400 spectrometer between 190 and 330 K. The probe temperature was calculated from the chemical shift separation of the resonances of aliphatic and hydroxyl protons of methanol[195, 196].

The puckering equilibrium of the deoxyribose rings was analysed by the use of vicinal spin-spin coupling constants[186]. These values were used in the calculation of the differences in enthalpy and entropy between the favoured conformations adopted over the range of temperatures studied. When measuring the coupling constants for fluorinated deoxyribose moieties, it was assumed, where necessary, that the corresponding $J_{H,H} = 1/2\,(J_{H,F})$[197]. This adjustment of coupling constants is not required for the ΔH and ΔS calculations, but such a correction factor is required to estimate

a value for the percentage of N- and S-character in the conformations adopted by fluorinated nucleosides.

4.4: EXPERIMENTAL

4.4.1: Synthesis of 1-(2′-deoxy-β-D-*erythro*-pentofuranosyl)thymine (31)[73]

4.4.1.1: Synthesis of 5′-methoxytritylthymidine (28)

Thymidine (5.0g, 20.6mmol) and DMAP (10mg, catalytic amount) were dissolved in dry pyridine (30ml) and cooled to 0°C in an ice-water bath. To the cooled solution was added p-anisylchlorodiphenylmethane (9.56g, 31.0mmol) . The mixture was stirred under nitrogen at room temperature overnight before being poured into ice-water (500ml) with vigorous stirring. The solution was stirred for 1 h and the precipitated white solid was collected by filtration through a glass sinter funnel, washed with water (50ml) and lyophilised to give 10.04g (95% yield) of white crystals: Rf = 0.29 MeOH/CH_2Cl, 1/9); ^1H NMR ($CDCl_3$) δ 1.47 (3H, d, J=1.14Hz, $C_{5'}$–Me), 2.32 (1H, ddd, J=2 x 6.92, 14.8Hz, $H_{2'a}$), 2.46 (1H, ddd, J=2.21, 6.09, 14.6Hz, $H_{2'b}$), 3.38 (1H, dd, J=3.89, 12.2Hz, $H_{5'a}$), 3.48 (1H, dd, J=2.92, 12.2Hz, $H_{5'b}$), 3.80 (3H, s, TrOMe), 4.11 (1H, ddd appears as quartet, J=3 x 3.52Hz, $H_{4'}$), 4.59 (1H, unresolved ddd, $H_{3'}$), 6.45 (1H, dd appears as t, J=6.65Hz, $H_{1'}$), 6.85-7.50 (14H, m, Tr), 7.64 (1H, d, J=1.17Hz, H_6), 9.12 (1H, s, NH); MS (EI) m/z (%) 43 (13), 55 (19), 69 (8), 81 (11), 98 (7), 126 (19, thymine$^{+\cdot}$), 152 (6), 165 (11), 197 (12), 229 (6), 273 (63); (CI) m/z (%) 55 (1), 81 (1), 99 (1), 127 [5, (thymine+H)$^+$], 153 (1), 167 (1), 197 (1), 243 (1), 273 (4), 274 (3), 292 (1), 515 [1, (M+H)$^+$].

4.4.1.2: Synthesis of 3′-methanesulphonyl-5′-methoxytritylthymidine (29)

5′-Methoxytritylthymidine (2.0 g, 3.9mmol) and DMAP (4 mg, catalytic amount) were dissolved in dry pyridine (20 ml). Methanesulphonyl chloride (2.0 ml, 9.3mmol) was added to the cooled solution at 0°C under nitrogen. The reaction mixture was stirred overnight at room temperature and then poured onto ice-water (200 ml), with vigorous stirring. The solution was stirred for 1 h before the precipitate was collected by filtration through a glass sinter funnel, and washed with water (10 ml). The white precipitate was dissolved in acetone, filtered and reduced *in vacuo* before being lyophilised to give 2.12 g (92% yield): Rf=0.51 ($MeOH/CH_2Cl_2$, 1/9); ^1H NMR ($CDCl_3$) δ 1.49 (3H, d, J=1.12Hz, C5′–Me), 2.53 (1H, ddd, J=2 x 6.95, 14.7Hz, $H_{2'a}$), 2.67 (1H, ddd, J=2.26, 6.12, 14.6Hz, $H_{2'b}$), 3.05 (3H, s, SO_2Me), 3.47 (1H, dd, J=3.84, 12.1Hz, $H_{5'a}$), 3.48 (2H, dd, J=2.93, 12.1Hz, $H_{5'b}$), 3.84 (3H, s, TrOMe), 4.32 (1H, ddd appears as quintet, J=2.96, 3.87, 6.84Hz, $H_{4'}$), 5.45 (1H, ddd, J=2 x 2.78, 6.80Hz, $H_{3'}$), 6.49 (1 H, dd, J=6.15, 7.99Hz, $H_{1'}$), 6.85-7.68 (14H, m, Tr), 7.88 (1H, d, J=1.14Hz, H_6), 9.70 (1H, s, NH); MS (EI) m/z (%) 55 (31), 81 (56), 93 (1), 126 (46, thymine$^{+\cdot}$), 165 (33), 229 (20), 273 (100); (CI) m/z (%) 55 (7), 81 (12), 127 [38, (thymine+H)$^+$], 165 (5), 225 (7), 273 (49), 497 (2), 593 [1, $(M+H)^+$].

4.4.1.3: Synthesis of 1-(5′-methoxytrityl-2′-deoxy-β-D-*erythro*-pentofuranosyl)thymine (30)

3′-Methanesulphonyl-5′-methoxytritylthymidine (2.12g, 3.6mmol) was dissolved in ethanol (40ml). To the solution was added water (20ml) containing 1.0N NaOH (15ml) and the mixture was refluxed under nitrogen for 5 h. The solution was neutralised with 1.0N HCl and the volume was reduced *in vacuo* to ~5ml. The solution was extracted with

CH_2Cl_2 (2 x 20ml), water (150ml) was added and the solution was left to stand at 4°C. The precipitated crystals were separated by filtration, washed with water (20ml) and lyophilised before being purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 5/95) to give 1.38g (75% yield) of a white solid: ^1H NMR ($CDCl_3$) δ 1.79 (3H, s, C5′–Me), 2.12 (1H, ddd appears as m, $H_{2'a}$), 2.57 (1H, ddd, J=5.42, 8.31, 14.3Hz, $H_{2'b}$), 3.08 (1H, s, OH), 3.49 (1H, dd, J=5.94, 10.2Hz, $H_{5'a}$), 3.62 (1H, dd, J=4.85, 10.2Hz, $H_{5'b}$), 3.79 (3H, s, TrOMe), 4.02 (1H, ddd appears as quartet, J=3 x 5.08Hz, $H_{4'}$), 5.41 (1H, unresolved ddd, $H_{3'}$), 6.20 (1 H, dd, J=2.35, 8.32Hz, $H_{1'}$), 6.81-7.46 (14H, m, Tr), 7.66 (1H, s, H_6), 8.66 (1H, s, NH).

4.4.1.4: Synthesis of 1-(2′-deoxy-β-D-*erythro*-pentofuranosyl)thymine (31)

1-(5′-Methoxytrityl-2′-deoxy-β-D-*erythro*-pentofuranosyl)thymine (1.30g, 2.5mmol) was dissoved in 50% acetic acid (20ml) and refluxed for 20 min under nitrogen to remove the methoxytrityl group. The solvent was removed *in vacuo* and the residue was lyophilised before being purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 5/95) to give 0.51g (83% yield) of a white solid: ^1H NMR (CD_3OD) δ 1.92 (3H, d, J=1.20Hz, C5′–Me), 2.05 (1H, ddd, J=1.00, 2.55, 14.9Hz, $H_{2'a}$), 2.68 (1H, ddd, J=5.38, 8.25, 14.9Hz, $H_{2'b}$), 3.92 (1H, dd, J=6.65, 11.56Hz, $H_{5'a}$), 3.95 (1H, dd, J=2.58, 11.6Hz, $H_{5'b}$), 4.00 (1H, ddd appears as septet, J=3.21, 4.32, 6.68Hz, $H_{4'}$), 4.43 (1H, ddd, J=0.93, 3.09, 5.36Hz, $H_{3'}$), 6.19 (1H, dd, J=2.52, 8.24Hz, $H_{1'}$), 7.98 (1H, d, J=1.22Hz, H_6); MS (EI) m/z (%) 45 (65), 55 (100), 73 (80), 81 (34), 99 (66), 110 (45), 117 (99), 126 (93, thymine$^{+\cdot}$), 127 (72), 153 (16), 242 (15, $M^{+\cdot}$); (CI) m/z (%) 55 (5), 81 (20), 98 (10), 99 (11), 117 (11), 127 [42, (thymine+H)$^+$], 144 (5), 153 (2), 207 (1), 243 [26, (M+H)$^+$].

4.4.2: Synthesis of 3′-fluoro-3′-deoxythymidine (32)

4.4.2.1: Synthesis of 5′-methoxytrityl-3′-fluoro-3′-deoxythymidine

1-(5′-Methoxytrityl-2′-deoxy-β-D-*erythro*-pentofuranosyl)thymine (1.40g, 2.7mmol) was dissolved in dry CH_2Cl_2 (20ml) and the mixture was cooled to 0°C in an ice-water bath. To the cooled solution, DAST (1.3ml, 9.85mmol) was added slowly and the solution stirred for 3 h at room temperature. The solution was poured into sodium bicarbonate solution (20ml), extracted with CH_2Cl_2 (2 x 20ml), dried over $MgSO_4$, filtered and evaporated *in vacuo*. The residue was purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 5/95) to give 0.42g (30% yield) of a white solid: ^1H NMR ($CDCl_3$) δ 1.43 (3H, s, C5′–Me), 2.36 (1H, dddd, J=4.89, 9.39, 14.33, 39.77Hz, $H_{2'a}$), 2.69 (1H, dddd, J=2 x 5.55, 14.50, 20.18Hz, $H_{2'b}$), 3.40 (1H, dd, J=3.31, 11.9Hz, $H_{5'a}$), 3.95 (1H, dd, J=3.07, 11.9Hz, $H_{5'b}$), 3.81 (3H, s, TrOMe), 4.37 (1H, dt, J=3.14, 27.75Hz, $H_{4'}$), 5.32 (1H, dd, J=4.65, 53.82Hz, $H_{3'}$), 6.53 (1H, dd, J=5.46, 9.37Hz, $H_{1'}$), 7.65 (1H, s, H_6), 9.55 (1H, s, NH).

4.4.2.2: Synthesis of 3′-fluoro-3′-deoxythymidine (32)

5′-Methoxytrityl-3′-fluoro-3′-deoxythymidine (0.40g, 0.78mmol) was dissolved in 50% acetic acid (10ml) and stirred at room temperature for 50 min under nitrogen to remove the methoxytrityl group. The solvent was removed *in vacuo* and the residue was lyophilised before being purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 5/95) to give 0.10g (53% yield) of a white solid: ^1H NMR (CD_3OD) δ 1.92 (3H, d, J=1.21Hz, C5′–Me), 2.28 (1H, dddd, J=4.96, 9.39, 14.32, 39.04Hz, $H_{2'a}$), 2.37 (1H, dddd, J=2 x 5.56, 14.46, 20.85Hz, $H_{2'b}$), 3.80 (1H, ddd, J=1.18, 3.36, 11.94Hz, $H_{5'a}$), 3.95 (1H, dd, J=3.05, 11.94Hz, $H_{5'b}$), 4.28 (1H, dt, J=2 x 3.24, 27.54Hz, $H_{4'}$), 5.15 (1H, dd,

J=4.90, 53.91Hz, $H_{3'}$), 6.36 (1H, dd, J=5.53, 9.25Hz, $H_{1'}$), 7.87 (1H, d, J=1.23Hz, H_6); HRMS (EI) calcd ($C_{10}H_{13}N_2O_4F^{+\cdot}$) 244.0859, found 244.0861.

4.4.3: Synthesis of 3′,3′-difluoro-3′-deoxythymidine (35)[190]

4.4.3.1: Synthesis of 3′-keto-5′-tritylthymidine (34)

A slurry of 4Å molecular sieve powder (1.50g) and pyridinium dichromate (1.50g, 3.99mmol) in CH_2Cl_2 (10ml) was magnetically stirred at room temperature. The flask was initially flushed with nitrogen and then maintained under an atmosphere of argon. 5′-Tritylthymidine (1.25g, 2.6mmol) was added in CH_2Cl_2 (10ml) and the colour of the reaction mixture changed from light orange to dark brown within five minutes. The reaction was stirred for 2 h, then filtered on a Büchner funnel through a celite pad. The collected solids were washed with CH_2Cl_2 (2 x 20ml) and the filtrate was evaporated *in vacuo* to give a brown solid which was suspended in EtOAc (200ml) by sonicating the mixture for a few minutes. The suspension was filtered through 4Å molecular sieve powder (deposited as a slurry with EtOAc) on a glass sinter funnel and the solids were washed with EtOAc (25ml). The solvent was evaporated *in vacuo* to yield 1.03g of a light tan powder. The crude solid was recrystallised from CH_2Cl_2/diethyl ether, 1/3 and the white crystals were separated by filtration and lyophilised to yield and first crop of 0.60g and a second crop of 0.31g (73% yield): Rf=0.32 for the product and 0.71 due to decomposition which yielded 2-trityloxymethyl-3(2H)-furanone (MeOH/CH_2Cl_2, 5/95); ^{1}H NMR ($CDCl_3$) δ 1.31 (3H, d, J=1.14Hz, C5′–Me), 2.76 (1H, dd, J=8.25, 18.68Hz, $H_{2'a}$), 3.08 (1H, dd, J=6.69, 18.65Hz, $H_{2'b}$), 3.41 (1H, dd, J=2.30, 10.43Hz, $H_{5'a}$), 3.67 (1H, dd, J=2.59, 10.43Hz, $H_{5'b}$). 4.17 (1H, dd appears as br t, $H_{4'}$), 6.57 (1 H, dd, J=6.70, 8.21Hz, $H_{1'}$), 7.23-7.37 (15H, m, Tr), 7.63 (1H, d, J=1.28Hz, H_6),

8.61 (1H, brs, NH); MS (EI) m/z (%) 52 (21), 63 (32), 71 (10), 89 (68), 107 (66), 136 (93), 154 (100), 165 (27), 243 (74, Trityl$^{+\cdot}$), 289 (8), 391 (4), 482 (4, M$^{+\cdot}$).

4.4.3.2: Synthesis of 3′,3′-difluoro-3′-deoxythymidine (35)

3′-Keto-5′-tritylthymidine (0.57g, 1.18mmol) was dissolved in CH_2Cl_2 (20ml) and the flask was purged with nitrogen. DAST (0.48g, 0.39ml, 4.14mmol) was added dropwise to the solution and the mixture was stirred for 24 h under nitrogen at room temperature. The solution was poured into a saturated aqueous solution of sodium bicarbonate (30ml) and then extracted with sodium bicarbonate (2 x 30ml), dried over $MgSO_4$ and evaporated *in vacuo* to yield a yellow residue. The residue was lyophilised before being purified by flash chromatography on silica gel (MeOH/CH_2Cl_2, 5/95) to give 0.18g (31% yield) of a pale yellow solid: Rf=0.55 (MeOH/CH_2Cl_2, 5/95).

The 5′-trityl-3′,3′-difluoro-3′-deoxythymidine (0.18g, 0.38mmol) was dissolved in 50% acetic acid (10ml) and the mixture was refluxed for 15 min. The solution was evaporated *in vacuo* and lyophilised before being purified by flash chromatography on silica gel (MeOH/CH_2Cl_2, 2/98) to give 0.07g (74% yield) of a pale yellow solid: Rf=0.38 (MeOH/CH_2Cl_2, 1/9); ^{1}H NMR (CD_3OD) δ 1.93 (3H, d, J=1.19Hz, C5′–Me), 2.67 (1H, dddd, J=8.13, 13.60, 14.3, 20.6Hz, $H_{2'b}$), 2.86 (1H, dddd, J=2 x 6.78, 14.17, 14.3Hz, $H_{2'a}$), 3.85 (1H, ddd, J=1.43, 4.10, 12.5Hz, $H_{5'a}$), 3.90 (1H, dd, J=3.58, 12.5Hz, $H_{5'b}$), 4.28 (1H, dddd, J=2 x 3.74, 7.37, 15.79Hz, $H_{4'}$), 6.35 (1H, dd, J=6.46, 8.13Hz, $H_{1'}$), 7.87 (1H, d, J=1.25Hz, H_6); ^{19}F NMR (CD_3OD) δ -98.16 (1F, dddd, J=2 x 15.14, 21.41, 238.44Hz, F_b), -109.89 (1F, dddd, J=2 x 7.3, 13.7, 238.44Hz, F_a); MS (EI) m/z (%) 55 (72), 69 (20), 83 (43), 87 (76), 93 (20), 117 (74), 126 (100, thymine$^{+\cdot}$),

188 (23), 231 (11), 262 (12, $M^{+\cdot}$); (CI) m/z (%) 55 (6), 83 (2), 99 (6), 117 (5), 126 (26), 127 [33, $(thymine+H)^+$], 188 (1), 263 [57, $(M+H)^+$].

4.4.4: Synthesis of 2′-fluoro-2′-deoxyuridine (39)

4.4.4.1: Synthesis of 2,2′-anhydro-1-(β-D-*arabino*-furanosyl)cytosine hydrochloride (37)[191]

A suspension of cytidine (1.85g, 7.59mmol) and 2-acetoxyisobutyryl chloride (5.0g, 30.0mmol) in CH_3CN (20ml) was stirred at 80°C under nitrogen. After 3 min the solution cleared and this was shortly followed by the formation of some fine white crystals. After 30 min the mixture was cooled to room temperature and ether (50ml) was added to the stirred solution. The solid was collected by filtration, washed with ether (2 x 10ml) to yield 0.88g of crude product. The filtrate was evaporated *in vacuo* and the solid was redissolved in ether (20ml) and left at 4°C to crystallise a further 1.41g of crude product: total 2.29g (99% yield).

The crude product (2.20g, 7.24mmol) was dissolved in methanol (60ml) containing conc. HCl (1ml) and stirred under nitrogen at room temperature for 6 days during which time crystals of product separated. The crystals were separated by filtration to yield 1.10g and the filtrate was evaporated *in vacuo*. The solid was recrystallised from MeOH/acetone to give a total of 1.55g (82% yield) of white crystals: Rf=0.07 ($MeOH/CH_2Cl_2$, 1/9); m.p.=264-265°C; ^1H NMR (d_6-DMSO) δ 3.27 (1H, dd, J=3.27, 12.03Hz, $H_{5'a}$), 3.40 (1H, dd, J=2.85, 12.12Hz, $H_{5'b}$), 4.22 (1H, unresolved ddd, $H_{4'}$), 4.47 (1H, unresolved dd, $H_{3'}$), 5.06 (1H, brs, OH), 5.39 (1H, d, J=6.02Hz, $H_{2'}$), 6.16 (1H, brs, OH), 6.54 (1H, d, J=6.02Hz, $H_{1'}$), 6.60 (1H, d, J=7.32Hz, H_5), 8.28 (1H, d, J=7.29Hz, H_6), 9.21 (1H, brs, NH), 9.53 (1H, brs, NH); MS (EI) m/z (%)

55 (85), 69 (100), 81 (67), 97 (54), 112 (31), 135 (35); (CI) m/z (%) 69 (3), 81 (7), 96 (5), 112 (5), 113 (34), 136 (10), 226 (3), 262 [1, $(M+H)^+$].

4.4.4.2: Synthesis of 2′-fluoro-2′-deoxycytidine (38)[192]

A mixture of DMF (50ml), dicyclohexano-18-crown-6 (0.5ml, 1.34mmol), KF (1.25g, 21.6mmol, dried over P_2O_5,100°C/0.01torr) and benzene (50ml) was heated at 135°C and the benzene was slowly distilled off. On completion of distillation, 2,2′-anhydrocytidine hydrochloride (0.5g, 1.92mmol) was added and the mixture was heated at 120°C under nitrogen for 5 h. The solvent was evaporated *in vacuo* and the pale brown oil was purified by flash chromatography on silica gel (MeOH/CH_2Cl_2, 1/5) to give 0.19g (41% yield) of white crystals: Rf=0.38 (MeOH/CH_2Cl_2, 3/7); m.p.=165-167°C; ^{1}H NMR (d6-DMSO) δ 3.58 (1H, dd, J=2.79, 12.4Hz, $H_{5'a}$), 3.73 (1H, dd, J=3.01, 12.4Hz, $H_{5'b}$), 4.87 (1H, unresolved ddd, $H_{4'}$), 4.10 (1H, ddd, J=4.65, 7.46, 22.44Hz, $H_{3'}$), 4.86 (1H, ddd, J=1.50, 4.35, 53.32Hz, $H_{2'}$), 5.72 (1H, d, J=7.46Hz, H_5), 5.87 (1H, dd, J=1.53, 17.99Hz, $H_{1'}$), 7.89 (1H, d, J=7.46Hz, H_6); MS (EI) m/z (%) 55 (15), 69 (10), 81 (6), 97 (4); (CI) m/z (%) 61 (1), 85 (3), 96 (1), 111 (1), 139 (1), 246 [1, $(M+H)^+$].

4.4.4.3: Synthesis of 2′-fluoro-2′-deoxyuridine (39)[193]

2′-Fluoro-2′-deoxycytidine (30mg, 0.12mmol) was dissolved in water (10ml) containing sodium metabisulphite (12mg, 1.2mmol) and the mixture was stirred under nitrogen overnight at room temperature. Unreacted sodium bisulphite was precipitated by the addition of barium hydroxide (3.15g, 10.0mmol) and the white solid was separated by filtration. The filtrated was purified by ion exchange chromatography on Dowex 50W H^+ and the uridine nucleoside was eluted with distilled

water. The eluted aqueous fractions were concentrated *in vacuo* and lyophilised before being purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 1/9) to give 22mg (73% yield) of white crystals: Rf=0.13 ($MeOH/CH_2Cl_2$, 2/8); m.p.=165-167°C; ^1H NMR (CD_3OD) δ 4.24 (1H, ddd, J=2.41, 2.95, 7.3Hz, $H_{4'}$), 4.25 (1H, dd, J=2.95, 12.0Hz, $H_{5'a}$), 4.39 (1H, ddd, J=4.39, 7.26, 18.75Hz, $H_{3'}$), 4.42 (1H, dd, J=2.41, 11.86Hz, $H_{5'b}$), 5.08 (1H, ddd, J=2.31, 4.43, 52.62Hz, $H_{2'}$), 5.78 (1H, d, J=8.13Hz, H_5), 6.06 (1H, dd, J=2.30, 16.50Hz, $H_{1'}$), 7.99 (1H, d, J=8.14Hz, H_6); HRMS (EI) calc ($C_9H_{11}N_2O_5F^{+\cdot}$) 246.05077, found 246.0509.

4.4.5: Synthesis of (±)-1-[(2β′,4β′)-2′-(hydroxymethyl)-4′-dioxolanyl] thymine. [(±)-Dioxolane–T] (50)[184]

4.4.5.1: Synthesis of (±)–methyl glycerate (44)

To a stirred suspension of (±)–calcium glycerate dihydrate (25.0g, 0.087mol) in methanol (500ml) was added 96% H_2SO_4 (10.0ml, 0.180mol) over a 2 min period. After 24 h at 25°C, a trace of methyl orange was added, the reaction mixture cooled to 0°C, and then rapidly neutralised with 50% sodium hydroxide to give an apparent pH of 6.0–6.5 (a colour change of pink to yellow). The solid material was removed by filtration through a celite pad which was washed with methanol (500ml). The combined organic fractions were reduced *in vacuo* before Kugelrohr distillation (150°C/0.1mm Hg) to afford 17.98g (86% yield) of product as a clear oil: ^1H NMR (CD_3OD) δ 3.79 (3H, s, OMe), 3.81 (2H, dd, J=3.97, 4.66Hz, CH_2), 4.26 (1H, t, J=4.30Hz, CH); ^{13}C NMR (CD_3OD) 52.49 (OMe), 65.00 (CH_2), 73.28 (CH), 174.53 (CO); HRMS (CI) calcd ($C_4H_8O_4^+ + H^+$) 121.0501, found 121.0500.

4.4.5.2: Synthesis of (±)–Methyl-2-benzyloxymethyldioxolane-4-carboxylate (45)

Benzyloxyacetaldehyde methyl acetal (24.53g, 0.125mol), (±)–methyl glycerate (**44**) (17.90g, 0.149mol) and p–toluene sulphonic acid (0.77g, 4.03mmol) in CH_3CN (50ml) were refluxed for one hour, after which 20% of the total volume was distilled off over a period of 45 min. The remaining solution was refluxed for a further 2 h, concentrated *in vacuo*, dissolved in CH_2Cl_2 (50ml), extracted with $NaHCO_3$ (2 x 30ml) and dried over $MgSO_4$. The organic layer was separated by filtration and concentrated *in vacuo* to give 23.34g (74% yield) of a pale brown oil which was used without further purification: ^1H NMR ($CDCl_3$) racemic 1:1 mixture of diastereomers δ 3.57-3.72 [2H, m, $H_{5'a\&b}(\alpha \& \beta)$], 3.69 & 3.73 [3H, 2 x s, OMe(α & β)], 4.05 [1H, 2 x dd, J=5.34, 8.26Hz & 7.36, 8.61Hz, $H_{2'a}(\alpha \& \beta)$], 4.23 [1H, 2 x dd, J=3.76, 8.56Hz & 2 x 7.27Hz, $H_{2'b}(\alpha \& \beta)$], 4.54–4.65 [3H, m, $H_{4'}(\alpha \& \beta)$ & C_6H_5–$\underline{C}H_2$–O], 5.15–5.34 [1H, 2 x dd, J=2 x 3.61Hz & 2 x 4.28Hz, $H_{1'}(\alpha \& \beta)$], 7.22–7.41 [5H, m, $C_6H_5(\alpha \& \beta)$[; ^{13}C NMR ($CDCl_3$) 51.94 (OMe), 67.77, 68.19 [$C_{2'}(\alpha \& \beta)$], 69.84, 70.42 [$C_{5'}(\alpha \& \beta)$],73.19, 73.23, 73.37, 73.52 [$C_{4'}(\alpha \& \beta)$ & C_6H_5–$\underline{C}H_2$–O], 103.82, 104.35 [$C_{1'}(\alpha \& \beta)$], 127.31–127.99 (C_6H_5); HRMS (EI) calcd ($C_{13}H_{16}O_5^{+\cdot}$) 252.1004, found 252.1001.

4.4.5.3: Synthesis of (±)-2-benzyloxymethyldioxolane-4-carboxylic acid (46)

Dioxolane methyl ester racemate (**45**) (23.0g, 0.0912mol) and LiOH (4.28g, 0.102mol) in THF/H_2O (90ml, 4/1) were refluxed for 1.5 h. The solvent was removed *in vacuo* and the oil was redissolved in CH_2Cl_2 (50ml), extracted with 1.0M HCl (30ml), H_2O (30ml) and dried over $MgSO_4$. The combined organic fractions were concentrated *in vacuo* to give 11.14g (51% yield) of a pale brown oil which was used without further purification: ^1H NMR

($CDCl_3$) 1:1 mixture of diatereomers δ 3.56 [1H, unresolved dd, $H_{5'a}$(α & β)], 3.72 [1H, unresolved dd, $H_{5'b}$(α & β)], 3.98 & 4.05 [1H, 2 x dd, J=2 x 7.47Hz & 2 x 6.25Hz, $H_{2'a}$(α & β)], 4.28 [1H, 2 x dd, J=2 x 7.26Hz & 2 x 7.72Hz, $H_{2'b}$(α & β)], 4.57–4.70 [3H, m, $H_{4'}$(α & β) & C_6H_5–$\underline{CH_2}$–O], 5.14–5.30 [1H, 2 x br t, J=2 x 3.43Hz & unres., $H_{1'}$(α & β)], 7.21–7.35 (5H, m, C_6H_5), 10.05 (1H, brs, OH); ^{13}C NMR ($CDCl_3$) 67.94, 68.22 [$C_{2'}$(α & β)], 70.07, 70.15 [$C_{5'}$(α & β)], 73.67 (C_6H_5–$\underline{C}H_2$–O), 74.06 [$C_{4'}$(α & β)], 104.14, 104.20 [$C_{1'}$(α & β)], 127.81–128.66 (C_6H_5); HRMS (EI) calcd ($C_{12}H_{14}O_5^{+\cdot}$) 238.0853, found 238.0847.

4.4.5.4: Synthesis of (±)-4-acetoxy-2-benzyloxymethyldioxolane (47)

Dioxolane carboxylic acids (**46**) (6.5g, 27.3mmol), lead tetraacetate (15.57g, 35.0mmol), and pyridine (2.37g, 30.0mmol) in CH_3CN (100ml) were stirred at 40°C under nitrogen for 6 h. The solution was concentrated *in vacuo* and lyophilised before being purified by flash chromatography on silica gel ($MeOH/CH_2Cl_2$, 2/98) to give 5.54g (80% yield) of the title compound as a clear oil: Rf = 0.86 ($MeOH/CH_2Cl_2$, 1/9), ^1H NMR ($CDCl_3$) 1.7:1.0 mixture of cis and trans isomers δ 1.94 [3H, s, Me (α-form)], 2.00 [3H, s, Me (β-form)], 3.53 [2H, 2 x ddd, J=1.30, 3.83, 0.51, 3.99, 11.06Hz, $H_{5'a\&b}$(β-form)], 3.59 [2H, dd, J=2.79, 4.24Hz, $H_{5'a\&b}$(α-form)], 3.86 [1H, m, $H_{2'a}$(β-form)], 3.90 [1H, m, $H_{2'a}$(β-form)], 4.13 [1H, d, J=10.00Hz, $H_{2'b}$(β-form)], 4.17 [1H, dd, J=4.43, 9.29Hz, $H_{2'b}$(α-form)],4.55 [2H, s, C_6H_5–$\underline{CH_2}$–O(β-form)], 4.57 [2H, s, C_6H_5–$\underline{CH_2}$–O(α-form)], 5.24 [1H, dd appears as t, J=2 x 4.22Hz, $H_{4'}$(α-form)], 5.34 [1H, dd appears as t, J=2 x 3.67Hz, $H_{4'}$(β-form)], 6.27 [1H, d, J=3.66Hz, $H_{1'}$(α-form)], 6.35 [1H, dd, J=2.08, 4.42Hz, $H_{1'}$(β-form)], 7.20–7.35 (5H, m, C_6H_5); ^{13}C NMR ($CDCl_3$) 13.30 [Me(β-form)], 13.35 [Me(α-form)], 62.43 [$C_{5'}$(β-form)], 62.97 [$C_{2'}$(β-form)], 63.34 [$C_{5'}$(α-form)], 63.47 [$C_{2'}$(α-form)], 65.77, 65.89 [C_6H_5–$\underline{C}H_2$–O(α & β-form)],86.23 [$C_{1'}$(α-form)], 86.73 [$C_{1'}$(β-form)], 95.99 [$C_{4'}$($C_{4'}$(β-form)],97.45 [$C_{4'}$(α-form)], 120.01–120.70 (C_6H_5), 162.41

[CO(β-form)], 162.45 [CO(α-form)]; MS (EI) m/z (%) 43 (89), 51 (11), 65 (33), 77 (16), 91 (100), 107 (47), 131 (82), 149 (7), 162 (8), 192 (19), 207 (10), 219 (4), 237 (6), 251 (1), 252 (1, $M^{+\cdot}$); (CI) m/z (%) 60 (2), 77 (1), 91 (8), 106 (5), 120 (2), 131 (3), 138 (1), 150 (3), 161 (1), 168 (2), 198 (3), 210 (5), 253 [3, $(M+H)^+$].

4.4.5.5: Synthesis of trimethylsilylthymine[61]

Thymine (1.0g, 7.9mmol), hexamethyldisilazane (8ml) and ammonium sulphate (4mg) were refluxed together under nitrogen for 16 h. Unreacted hexamethyldisilazane was removed *in vacuo* and the residue was co-evaporated twice with toluene (5ml). This compound was used immediately in the following coupling reaction without identification.

4.4.5.6: Synthesis of (±)-1-(2′-benzyloxymethyl-4′-dioxolanyl)thymine (48 and 49)[61]

The acetoxy compounds (**47**) (1.03g, 4.08mmol), trimethylsilylthymine (2.14g, 7.94mmol) and trimethylsilyl triflate (0.29g, 0.25ml, 1.29mmol) in dry CH_3CN (10ml) were stirred under nitrogen at room temperature for 1 week. The solution was concentrated *in vacuo* and the residue redissolved in CH_2Cl_2 (20ml), extracted with $NaHCO_3$ (2 x 20ml), dried over $MgSO_4$. The organic layer was concentrated *in vacuo* and lyophilised before being purified by flash chromatography on silica gel to separate the 1:1 mixture of α– and β–anomers of the title compound [EtOAc/petroleum ether(40-60), 7/3] to give a total of 1.01g (82% yield) of a white solid:

Rf = 0.11 (β–anomer) (**49**) [EtOAc/petroleum ether(40-60), 7/3]; ^1H NMR ($CDCl_3$) β–isomer, δ 1.61 (3H, d, J=1.10Hz, $C_{5'}$–Me), 3.81 (2H, d, J=2.24Hz, $H_{5'a\&b}$), 4.09 (1H, dd, J=5.21, 10.05Hz, $H_{2'a}$), 4.16 (1H, dd, J=1.86, 10.05Hz,

$H_{2'b}$), 4.61 (2H, s, C_6H_5-$\underline{CH_2}$–O), 5.07 (1H, dd appears as t, J=2 x 2.24Hz, $H_{4'}$), 6.36 (1H, dd, J=1.86, 5.21Hz, $H_{1'}$), 7.24–7.33 (5H, m, C_6H_5), 7.62 (1H, d, J=1.12Hz, H_6), 9.54 (1H, brs, NH); HRMS (EI) calcd ($C_{16}H_{18}N_2O_5$) 318.1216, found 318.1220.

Rf = 0.17 (α–anomer) (48), [EtOAc/petroleum ether(40-60), 7/3]; ^{1}H NMR ($CDCl_3$) α–anomer, δ 1.89 (3H, d, J=0.94Hz, $C_{5'}$–Me), 3.54 (2H, d, J=3.72Hz, $H_{5'a\&b}$), 4.00 (1H, dd, J=2.61, 9.68Hz, $H_{2'a}$), 4.37 (1H, dd, J=5.21, 9.68Hz, $H_{2'b}$), 4.58 (2H, s, C_6H_5-$\underline{CH_2}$–O), 5.57 (1H, dd appears as t, J=2 x 3.54Hz, $H_{4'}$), 6.28 (1H, dd, J=2.97, 5.58Hz, $H_{1'}$), 7.16 (1H, d, J=1.18Hz, H_6), 7.17–7.33 (5H, m, C_6H_5), 10.21 (1H, brs, NH); HRMS (EI) calcd ($C_{16}H_{18}N_2O_5$) 318.1216, found 318.1224.

4.4.5.7: Synthesis of (±)-1-[(2β′,4β′)-2′-(hydroxymethyl)-4′-dioxolanyl] thymine (50)

The (±)-1-(2′-benzyloxymethyl-4′-dioxolanyl)thymine (**49**) (0.17g, 0.54mmol) and $Pd(OH)_2$ (20% on C) (0.486g, 0.70mmol) in ethanol (30ml) were shaken at room temperature in a Parr bomb at 20psi of H_2 for 30 min. The catalyst was removed by filtration of the solution through a celite pad which was washed with ethanol (50ml). The solvent was removed *in vacuo* and lyophilised before being purified by flash chromatography on silica gel (CH_2Cl_2) to elute the toluene byproduct and ($MeOH/CH_2Cl_2$, 4/6) to give 0.11g (91% yield) of white crystals: Rf = 0.23 ($MeOH/CH_2Cl_2$, 1/9): m.p. = 149–150°C; ^{1}H NMR (CD_3OD) δ 1.91 (3H, d, J=1.07Hz, $C_{5'}$–Me), 3.85 (2H, 2 x dd, J=2.20, 2.21, 13.6Hz, $H_{5'a\&b}$), 4.18 (1H, dd, J=5.95, 10.42Hz, $H_{2'a}$), 4.31 (1H, dd, J=1.49, 10.05Hz, $H_{2'b}$), 5.03 (1H, dd appears as t, J=2 x 2.05Hz, $H_{4'}$), 6.35 (1H, dd, J=1.49, 5.59Hz, $H_{1'}$), 7.91 (1H, d, J=1.49Hz, H_6); ^{13}C NMR (CD_3OD) δ 12.53 (C_5-Me), 61.65 ($C_{5'}$), 72.10 ($C_{2'}$), 82.26 ($C_{1'}$), 106.63 ($C_{4'}$),

111.70 (C_5), 138.35 (C_6), 152.70 (C_2), 166.44 (C_4); HRMS (EI) calcd ($C_9H_{11}N_2O_5$) 228.0746, found 228.0737.

4.4.6: (±)-Dioxolane–T (50) as a Glycosyl Donor

4.4.6.1: N-Deoxyribosyltransferase

Five reactions were set-up with varying ratios of β-dioxolane-T and adenine in citrate buffer (50 mM, pH 6.0):

i) β-dioxolane-T (0.5mM) + adenine (0.5mM)

ii) β-dioxolane-T (1.0mM) + adenine (0.5mM)

iii) β-dioxolane-T (1.5mM) + adenine (0.5mM)

iv) β-dioxolane-T (2.5mM) + adenine (0.5mM)

v) β-dioxolane-T (5.0mM) + adenine (0.5mM)

The reactions were initiated by the addition of the crude enzyme preparation (50µl, 14.3mg ml^{-1} protein, 0.4U). The final volume of the reaction mixture was 0.5 ml and it was incubated at 40°C with no shaking. The reactions were followed by reverse phase HPLC on a Techsphere 5C8 column (25cm x 4.6 mm and a precolumn, 5cm x 4.6mm) as described in Chapter 2. No transfer was observed for any of the reactions.

4.4.6.2: Thymidine Phosphorylase

Three thymidine phosphorylases, from the organisms named below, were investigated to see if they were able to cleave β-dioxolane-T^{95}:

i) *Lactobacillus casei* (rhamnosus) NCIMB 6557

ii) *Lactobacillus casei* (rhamnosus) NCIMB 7473

iii) *Lactobacillus casei* (casei) NCIMB 4113

Each reaction contained β-dioxolane-T (10.0mM) and DTT (0.1mM) in sodium phosphate buffer (50 mM, pH 7.0) to a total volume of 0.5ml after the addition of a crude preparation of the thymidine phosphorylase (50µl). Each reaction was incubated at 40°C with no shaking and was followed by reverse phase HPLC on a Techsphere 5C8 column (25cm x 4.6 mm and a precolumn, 5cm x 4.6mm) as described in Chapter 2. No cleavage of the glycosyl bond and release of thymine was observed for any of the reactions.

APPENDIX 1: RELATIVE RATES OF GLYCOSYL TRANSFER

Table A.1.1: Relative Rates of Glycosyl Transfer between 2'-deoxycytidine and 2 and 6-substituted purine bases.

Compound	C-6	C-2	Relative Rates
Adenine	NH_2	H	1.00
6-Methyladenine	NHMe	H	0.76
2-Aminopurine	H	NH_2	0.60
6-Chloroguanine	Cl	NH_2	0.52
Purine	H	H	0.35
6-Chloropurine	Cl	H	0.27
Isoguanine	NH_2	=O	0.25
N-1 oxide adenine	NH_2	H	0.14
Hypoxanthine	=O	H	0.13
Xanthine	=O	=O	0.11
2-Hydroxypurine	H	=O	0.08
2,6-dichloropurine	Cl	Cl	0.07
2,6-diaminopurine	NH_2	NH_2	0.07

Reaction conditions:

2'-deoxycytidine (10mM) =	75ul
Purine Base (10mM) =	25ul
10% Ethylene Glycol =	50ul
Citrate Buffer(1mM,pH6.0) =	300ul
Crude Enzyme(36U) =	50ul
Total =	500ul

Reaction mixture thermostated at 40'C for 10 min before addition of the crude enzyme. A 20ul aliquot of the mixture was applied to a Techsphere 5C8 HPLC column at 0 min and again at 15 min. The initial rates were compared using the ratios of C:dC after the 15 min reaction time and were scaled so that the fastest transfer had a corresponding value of 1.00.

Table A.1.2: Relative Rates of Glycosyl Transfer between 2',3'-dideoxycytidine and 2 and 6-disubstituted purine bases.

Compound	C-6	C-2	Relative Rates
Adenine	NH_2	H	1.00
6-Methyladenine	NHMe	H	0.86
2-Hydroxypurine	H	=O	0.79
6-Chloropurine	Cl	H	0.78
Isoguanine	NH_2	=O	0.65
Purine	H	H	0.64
Hypoxanthine	=O	H	0.61
2-Aminopurine	H	NH_2	0.54
Xanthine	=O	=O	0.55
6-Chloroguanine	Cl	NH_2	0.45
2,6-dichloropurine	Cl	Cl	0.42
2,6-diaminopurine	NH_2	NH_2	0.36

Reaction conditions:

2',3'-dideoxycytidine (10mM) = 75ul
Purine Base (10mM) = 25ul
10% Ethylene Glycol = 50ul
Citrate Buffer(1mM,pH6.0) = 300ul
Crude Enzyme(180U) = 50ul

Total = 500ul

Reaction mixture thermostated at 40'C for 10 min before addition of the crude enzyme. A 20ul aliquot of the mixture was applied to a Techsphere 5C8 HPLC column at 0 min and again after 5 d. The transfer rates were compared using the ratios of C:ddC after the 5 d reaction time and were scaled so that the fastest transfer had a corresponding value of 1.00.

APPENDIX 2: VICINAL SPIN–COUPLING CONSTANTS

	J/Hz														
	H_6	$H_{1'}$		$H_{2'a}$		$H_{2'b}$			$H_{3'}$			$H_{4'}$	$H_{5'}$		
Temp /°C	H_6-Me	1′-2′a	1′-2′b	2′a-1′	2′a-3′	2′a-2′b	2′b-1′	2′b-3′	3′-2′a	3′-2′b	3′-4′	4′-3′	5′a-4′	5′a-5′b	5′b-4′
-64	0.99	6.91	6.91	6.70	4.1	13.3	6.7	4.4	3.8	4.4	2.45	2.70	2.88	12.2	3.03
-47	1.17	6.05	7.66	5.99	3.13	13.3	7.83	5.83	2.82	5.59	2.82	2.82	2.92	12.2	3.10
-36	1.13	6.80	6.80	—	—	13.3	—	5.3	—	—	—	3.00	2.99	12.1	3.28
+2	1.24	6.11	7.49	6.15	3.37	13.2	7.50	6.07	3.24	6.21	—	3.24	3.09	12.1	3.43
+25	1.23	6.80	6.80	6.39	3.84	13.2	7.29	5.85	3.84	5.76	3.3	3.35	3.18	12.0	3.66
+42	0.94	6.78	6.78	6.36	3.90	13.2	7.0	6.16	3.9	5.94	3.3	3.46	3.24	12.0	3.76

Table A.2.1 Coupling constants for thymidine (**1**)

	J/Hz														
	H_6	$H_{1'}$		$H_{2'a}$			$H_{2'b}$				$H_{3'}$		$H_{4'}$	$H_{5'}$	
Temp /°C	H_6-Me	1′-2′a	1′-2′b	2′a-1′	2′a-F	2′a-3′	2′b-1′	2′b-F	2′b-2′a	2′b-3′	3′-F	3′-4′	4′-F	5′a-5′b	5′b-4′
-70	1.08	9.84	5.22	9.99	42.74	4.16	4.34	19.73	14.3	0	52.96	0	28.49	2.24	2.28
-65	—	9.69	5.22	9.81	42.55	4.39	5.27	19.93	14.3	0	53.31	0	28.47	2.36	2.50
-14	1.07	9.60	5.31	9.76	41.52	4.61	5.39	20.12	14.4	0	53.52	0	28.16	2.66	3.00
+5	1.26	9.37	5.46	9.51	39.77	4.89	5.55	20.18	14.5	0	53.82	0	27.75	3.05	3.36
+24	1.23	9.25	5.53	9.39	39.04	4.96	5.56	20.85	14.5	0	53.91	0	27.54	3.07	3.41
+42	1.00	9.13	5.59	9.29	38.34	5.07	5.66	20.76	14.5	0	54.03	0	27.38	3.31	3.48

Table A.2.2 Coupling constants for 3′-fluoro-3′-deoxythymidine (**32**)

	J/Hz														
	H_6	$H_{1'}$		$H_{2'a}$		$H_{2'b}$			$H_{3'}$			$H_{4'}$	$H_{5'}$		
Temp /°C	H_6-Me	1′-2′a	1′-2′b	2′a-1′	2′a-3′	2′a-2′b	2′b-1′	2′b-3′	3′-2′a	3′-2′b	3′-4′	4′-3′	5′a-4′	5′a-5′b	5′b-4′
-65	—	6.33	6.33	5.97	6.53	13.7	5.97	6.53	4.95	5.34	5.34	4.49	2.74	12.4	2.47
-40	1.23	6.36	6.36	6.55	5.37	13.7	6.55	6.41	5.27	6.40	5.27	4.70	2.94	12.3	2.83
-14	1.23	6.38	6.38	6.58	5.39	13.7	6.58	6.48	5.15	6.75	5.15	4.79	3.08	12.2	3.08
+6	1.25	6.40	6.40	6.56	5.39	13.7	6.56	6.51	5.16	6.88	5.16	4.84	3.17	12.2	3.22
+29	1.20	6.43	6.43	6.54	5.40	13.8	6.54	6.51	5.17	6.93	5.17	4.85	3.27	12.2	3.38
+37	—	6.43	6.43	6.69	5.49	13.8	6.69	5.49	5.19	6.89	5.19	4.80	3.31	12.2	3.44

Table A.2.3 Coupling constants for 3′-azido-3′-deoxythymidine (**40**)

	J/Hz													
	H_6	$H_{1'}$		$H_{2'a}$		$H_{2'b}$			$H_{3'}$			$H_{4'}$		$H_{5'}$
Temp /°C	H_6-Me	1′-2′a	1′-2′b	2′a-1′	2′a-3′	2′a-2′b	2′b-1′	2′b-3′	3′-2′a	3′-2′b	3′-4′	4′-3′	4′-5′	5′b-4′
-39	—	6.78	6.78	6.47	4.28	14.3	6.70	6.70	4.06	5.92	4.06	3.90	6.26	—
+7	1.22	6.74	6.74	6.68	4.83	14.4	6.82	6.12	4.57	5.97	4.57	4.09	6.42	6.44
+23	1.16	6.71	6.71	6.69	5.96	14.5	6.69	5.96	5.37	4.20	4.20	4.16	6.41	6.41
+48	1.22	6.67	6.67	6.70	6.58	14.5	6.69	5.30	5.63	4.26	4.26	4.27	6.40	6.40

Table A.2.4 Coupling constants for 5′-deoxythymidine (**42**)

	J/Hz														
	H_6	$H_{1'}$		$H_{2'b}$			$H_{3'}$			$H_{4'}$	$H_{5'}$				
Temp /°C	H_6-Me	1′-F	1′-2′b	2′-1′	2′-F	2′-3′	3′-2′	3′-F	3′-4′	4′-3′	5′a-4′	5′a-F	5′a-5′b	5′b-4′	5′b-F
-65	—	16.76	3.90	3.70	52.40	2.90	2.76	19.64	4.82	4.10	3.58	—	13.6	5.84	—
-39	—	16.74	3.89	3.26	52.51	3.26	2.52	19.51	4.55	4.22	3.10	—	13.4	4.77	—
-14	1.38	16.85	3.96	3.95	52.44	2.71	2.73	19.49	4.77	4.77	3.42	1.19	12.3	5.06	—
+5	1.42	16.94	3.98	3.97	52.40	2.67	2.69	19.55	4.84	4.71	3.62	1.02	12.2	5.15	0.53
+26	1.40	17.03	3.97	3.99	52.45	2.64	2.65	19.60	4.85	4.88	3.74	1.24	12.2	5.16	0.64
+46	—	17.09	3.96	3.80	52.42	3.06	2.59	19.65	4.84	4.85	4.15	—	12.1	5.31	—

Table A.2.5 Coupling constants for 2′-deoxy-2′-fluoro-5-methyl-arabinosyluracil (FMAU) (**41**)

	J/Hz														
	H_6		$H_{1'}$		$H_{2'}$	$H_{3'a}$			$H_{3'b}$				$H_{5'a}$		
Temp /°C	6-5	6-F	1′-2′	1′-F	2′-F	3′a-2′	3′a-4′	3′a-F	3′a-3′b	3′b-2′	3′b-4′	3′b-F	5′a-4′	5′a-5′b	5′b-4′
-65	7.80	1.79	3.03	18.69	54.33	1.46	5.06	27.99	14.8	5.57	8.63	35.94	3.29	12.2	6.31
-40	8.14	1.84	3.19	18.22	54.42	1.90	5.22	28.23	14.8	5.55	8.51	34.64	3.42	12.1	6.16
-14	8.12	1.80	3.29	17.85	54.39	2.12	5.41	28.33	14.8	5.66	8.40	33.71	3.69	12.1	6.11
+6	8.13	1.81	3.36	17.62	54.47	2.26	5.54	28.40	14.8	5.74	8.35	33.00	3.85	12.1	5.96
+29	8.14	1.76	3.43	17.42	54.49	2.36	5.67	28.48	14.8	5.81	8.28	32.31	3.94	12.1	5.86
+37	8.15	1.78	3.44	17.35	54.50	2.39	5.70	28.49	14.8	5.83	8.25	32.12	3.98	12.0	5.83

Table A.2.6 Coupling constants for 1-(2',3'-dideoxy-2'-fluoro-β-D-*threo*-pentofuranosyl)uracil (**43**)

	J/Hz														
	H_6	$H_{1'}$		$H_{2'a}$		$H_{2'b}$			$H_{4'}$				$H_{5'}$		
Temp /°C	H_6-Me	1′-2′a	1′-2′b	2′a-3′a	2′a-3′b	2′a-2′b	2′b-3′a	2′b-3′b	4′-3′a	4′-3′b	4′-5′a	4′-5′b	5′a-4′	5′a-5′b	5′b-4′
-64	—	6.63	2.30	8.41	8.41	12.2	3.09	3.09	6.29	8.82	2.53	2.53	2.06	12.6	2.88
-47	—	6.61	2.73	8.02	8.02	12.2	—	—	6.55	8.49	2.50	2.50	2.22	12.6	3.07
-36	1.20	6.71	3.12	9.18	9.18	12.2	—	—	6.50	8.74	3.10	3.10	2.75	12.3	3.41
+2	1.20	6.70	3.26	9.11	9.11	12.2	6.29	6.29	6.68	8.70	3.34	3.34	2.85	12.3	3.63
+25	1.20	6.73	3.49	8.99	8.99	12.1	6.59	6.59	6.91	8.68	3.31	3.31	2.97	12.2	3.79
+42	1.11	6.66	3.59	8.70	8.70	12.1	7.59	7.59	7.29	7.29	3.57	3.57	3.09	12.1	3.95

Table A.2.7 Coupling constants for 3′-deoxythymidine (**6**)

	J/Hz													
	H_6	$H_{1'}$		$H_{2'a}$		$H_{2'b}$			$F_{3'}$	$H_{4'}$		$H_{5'a}$	$H_{5'b}$	
Temp /°C	H_6-Me	1′-2′a	1′-2′b	2′a-Fa	2′a-Fb	2′a-2′b	2′b-Fa	2′b-Fb	Fa-Fb	4′-Fa	4′-Fb	5′a-4′	5′a-5′b	5′b-4′
-67	—	9.01	6.03	11.0	23.5	15.0	3.0	15.0	—	3.72	18.0	2.42	12.6	2.42
-58	0.89	8.94	6.15	11.5	23.2	14.9	4.0	14.8	—	4.20	17.8	3.0	12.6	3.0
-42	1.15	8.75	6.16	12.0	22.9	14.6	5.0	14.6	—	5.19	17.48	3.04	12.6	3.2
-22	1.26	8.56	6.26	12.80	22.60	14.6	5.87	14.58	—	6.67	16.77	3.36	12.6	3.37
-4	1.26	8.39	6.34	12.92	21.64	14.6	6.31	14.57	—	6.62	16.33	3.49	12.6	3.63
+29	1.25	8.13	6.46	13.60	20.61	14.2	6.78	14.5	238.4	7.37	15.79	3.58	12.5	3.58
+44	1.02	7.54	6.94	14.03	19.67	14.4	7.60	14.5	—	7.93	15.42	3.65	12.5	4.06

Table A.2.8 Coupling constants for 3′,3′-difluoro-3′-deoxythymidine (**35**)

	J/Hz														
	H_6	$H_{1'}$		$H_{2'a}$			$H_{3'}$			$H_{4'}$			$H_{5'}$		
Temp /°C	H_6-Me	1′-F	1′-2′a	2′-1′	2′-F	2′-3′	3′-2′	3′-F	3′-4′	4′-3′	4′-5′a	4′-5′b	5′a-4′	5′a-5′b	5′b-4′
-63	8.1	1665	<1.0	<1.0	53.18	3.87	3.99	22.64	8.27	10.0	—	—	—	12.3	—
-38	8.1	16.33	1.19	1.0	52.56	3.84	4.09	21.04	8.09	9.87	—	—	2.32	12.3	—
-13	8.1	16.25	1.85	1.77	52.63	4.30	4.26	20.12	7.33	7.87	—	2.94	2.55	12.2	2.94
+4	8.1	16.45	2.14	2.13	52.63	4.26	4.34	19.52	7.27	—	—	2.61	2.85	12.2	—
+24	8.1	16.48	2.23	2.32	52.63	4.42	4.39	18.75	7.26	—	—	2.30	—	12.1	—
+34	8.1	16.50	2.34	2.33	52.60	4.46	4.50	18.52	7.23	—	—	—	3.03	12.1	2.44

Table A.2.9 Coupling constants for 2′-fluoro-2′-deoxyuridine (**39**)

	J/Hz														
	H_6	$H_{1'}$		$H_{2'a}$		$H_{2'b}$			$H_{3'}$			$H_{4'}$	$H_{5'}$		
Temp /°C	H_6-Me	1′-2′a	1′-2′b	2′a-1′	2′a-3′	2′a-2′b	2′b-1′	2′b-3′	3′-2′a	3′-2′b	3′-4′	4′-3′	5′a-4′	5′a-5′b	5′b-4′
-75	1.06	1.64	8.11	1.54	0	14.8	8.78	5.48	0	4.88	2.99	3.02	~5	—	6.76
-59	1.19	1.86	8.22	1.92	0	14.9	~8	~5	0	4.89	3.01	2.95	4.64	12.8	6.84
-42	1.20	1.99	8.25	2.08	0	14.9	8.31	5.20	0	4.89	3.06	3.04	4.51	—	6.93
-24	1.21	2.17	8.28	2.24	0	14.9	8.32	5.23	0	4.90	3.24	3.12	4.45	11.7	6.95
-5	1.22	2.31	8.27	2.38	0.79	14.9	8.20	5.28	1.05	4.61	3.13	3.12	4.41	11.7	6.87
+23	1.22	2.52	8.24	2.55	1.0	14.9	8.25	5.38	0.93	5.36	3.09	3.12	4.32	11.6	6.68

Table A.2.10 Coupling constant for 1-(2'-deoxy-β-D-*erythro*-pentofuranosyl)thymine (**3**)

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